The Design Synthesis and Antimalarial Activity of Novel Diamidines, Diguanidines, Substituted Fluorenes and Their Prodrugs

Thesis submitted in accordance with the requirements of the University *of* Liverpool for the degree of Doctor in Philosophy

By

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BSc (Hons), MSc (Dist*)

September, 2008

Abstract

The aim of this thesis was to develop novel antimalarial drug candidates. This was achieved through drug design, targeting established parasite survival mechanisms. The project focused on the biomineralisation of hematin to hemozoin, a process critical for parasite survival (Figure A). In order to impart selectivity over host cells, functionalities shown to accumulate *via* parasite induced permeation pathways were exploited.

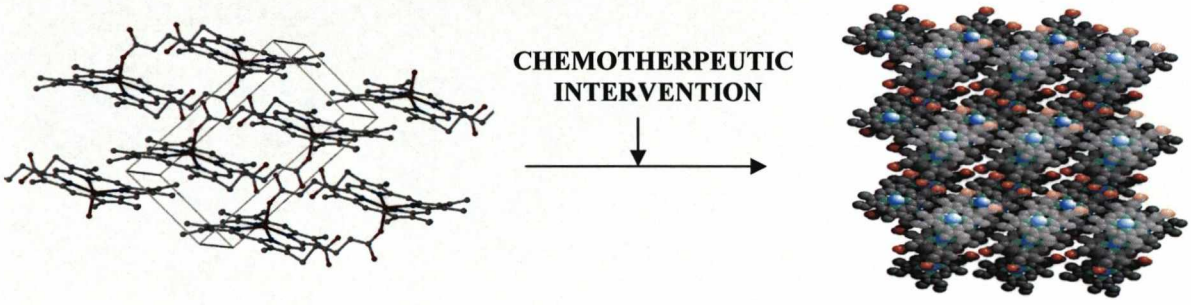


Figure A. Hematin crystallisation to non-toxic hemozoin

Chapter I provides a brief overview of vector borne infectious disease, discussing malaria epidemiology and chemotherapy. The thesis covers two drug forms consisting of *mono*-cationic and dicationic drug templates hence Chapter II is divided into two parts. Part I reviews dications and their consignment within antiplasmodial drug design. Part II covers the polyaromatic hydrocarbon fluorene; its chemistry and medicinal properties are introduced since fluorene forms the basis of the *mono*-cationic drug template.

A novel series of dicationic diamidines and diguanidines were synthesised. A novel diphenylthiazole compound was found to be the most active analogue, with an IC_{50} of 6.2 nM against chloroquine resistant parasites, making this compound more active than DB75 (75 nM). Chapter III describes the synthetic preparation of diamidines and diguanidines, along with the challenges encountered through the project. The design of these compounds was based on structure activity relationships obtained for the anti-protozoal agent pentamidine. The latter part of this chapter focuses on fluorene as a heme binding unit for *mono*-cationic antimalarials. This template was designed based on the antimalarials chloroquine, lumefantrine and amodiaquine. Referred to as Fluorene-Mannich Hybrids, these compounds are potent antimalarials and strong inhibitors of hemozoin formation with the lead compound having an IC_{50} of 12.7 nM, approximately three times more active than lumefantrine.

EPSRC

Acknowledgements

I can't believe it is time for me to write the acknowledgements page! Firstly I would like to thank *Prof. P. O'Neill* for the opportunity to work in such a vibrant area of research. I thank you for your support, positive comments and words of encouragement.....should there be a waffle alert here?!

I must extend a huge thank you to *Dr. P. Stocks*. I have enjoyed working with you; you were a good mentor, give good advice and support, for this I will always be thankful....delineate! *Gibbo*...who/what would you rather? Thanks for always being there, especially through my isoxazole rants!

Thank you to all members of the O'Neill group past and present – Louise, Vicky, Benedicte, Richard, Ally, (Dr Tobin!), Sunil, Robin, Chandra, David, Ian & Dr. S. Hindly, Debbie and Gemma. Special thanks must go to *Dr. J. Chadwick* for proof reading and words of support, *Dr. E. Row* for her help with HPLC, fluorene and thiazole chemistry and *J. Gaynor* for help with HPLC. Special thanks to the staff at the University *of* Liverpool and School of Tropical Medicine; Prof S. Ward, Dr P. Bray, Dr N. Greeves, Dr. N. Berry, Dr. P. Leonard, Dr A. Owen, J. Davies, C. Hall, K. Rimmer, A. Mills and A. Kaniti. Also Prof D. Taramelli (Università di Milano). Thank you to the Xiao group, I think I'm now an honouree member!

Thank you to my friends for their love and support. Big up Liz, Laura O, Han B, Hannah, Chi-Chi, Zoe, Andy and Mike x one in particular....*Zeyn*, without you there are so many points on this road that I would not have crossed, thanks for your strength, love and everlasting support. You are truly special to me x Paul; you arrived at the crucial writing up stage and have been such a good friend....what a diva!

I wish to thank *my family* for which I am truly blessed. Without the full support of my parents I would not have been able to fulfil my potential. I love you both dearly. To my sisters Natalie and Adaeze, no I don't use a Bunsen burner, yes why don't I become a mobile scientist! xxx To Granddad x Da Felicia, Da Stella, Da Holda and De Sam... Aga enwe Dr. Okpara Ọzọ na Amaigbo! Ugoeze, daalu O! Ada, it's finally finished...zup zup zup! x

Nana, Uncle Eric, Grandma and Granddad xxxxx

Definitions and Abbreviations

Δ	Reflux
%	Percent
ADME	Absorption Distribution Metabolism and Excretion
$AlCl_3$	Aluminium Chloride
ArH	Aromatic Hydrogen
Art	Artemisinin
ASPT1	Adenosine Sensitive PMD Transporter
AT	Adenine and Thymine
aq.	Aqueous
BBB	Blood Brain Barrier
bc 1	Mitochondrial Ubiquinol cytochrome c oxidoreductase
BMEA	Methoxyethylamine
Boc	*tert*-Butyloxycarbonyl
^{t}Bu	*tert*-Butyl
°C	Degree Celsius
^{13}C	Carbon-13
$CaCl_2$	Calcium Chloride
CCT	Choline Phosphate Cytidylyltransferase
$CDCl_3$	Deuterated Chloroform
CDP-Choline	Cytidine Diphosphate Choline
$CHCl_3$	Chloroform
Cl_2CHOMe	Dichloromethyl methyl ether
CH_3CN	Acetonitrile
CI	Chemical Ionisation
CK	Choline Kinase
CNS	Central Nervous System
Conc.	Concentrated
CPT	Choline Phosphotransferase
CQ	Chloroquine
CuCN	Copper (II) Cyanide
CYP4F	Cytochrome P450 enzyme of CYP4F family
DAG	Diacylglycerol
DAPI	4',6-Diamidino-2-phenylindole
DB75	Furamidine
DB289	Parfuramidine
DCM	Dichloromethane

DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
EI	Electron Impact
Et_2O	Diethylether
EtOAc	Ethylacetate
EtOH	Ethanol
$EtOH.NH_3$	Ethanol Ammonia Solution
ESP	Electrospray Ionisation
Factor VIIa	Protein within the blood coagulation process
F-M Hybrids	Fluorene Mannich Hybrids
Fmoc	(9-Fluorenylmethyl) carbamate
FPIX	Ferriprotoporphyrin IX
g	Gaseous
g	Gram
GC	Guanine and Cytosine
GSK	GlaxoSmithKline
H2	Histamine H2 receptor
H	Hydrogen
^1H	Proton
H_2S	Hydrogen Sulfide
HCl	Hydrogen Chloride
HF	Hydrogen fluoride
HIV	Human Immunodeficiency Virus
HAPT1	High Affinity PMD Transporter
$HgCl_2$	Mercury II chloride
HPLC	High Pressure Liquid Chromatography
hr.	Hour
IC_{50}	Concentration of drug inhibiting 50% biological response
ID_{50}	Dose required to reduce a given biological effect by 50%
IPA	Isopropyl alcohol
IPP	Induced Permeability Pathway
IR	Infra-red
i.v	Intravenous
ED_{50}	Effective Dose of Drug for 50% of Population
GI	Gastrointestinal
$KFAl_2O_3$	Aluminium Oxide Supported Potassium Fluoride
Kg	Kilogram

KJ	Kilojoules
KOH	Potassium Hydroxide
L	Litre
LAPT1	Low Affinity PMD Transporter
LapDap	Chloroproguanil-Dapsone
LED	Light Emitting Diode
$LiAlH_4$	Lithium Aluminium Hydride
Lu	Lumefantrine
M	Molar
m	meta
µm	micromole
MeCN	Acetonitrile
Me	Methyl
µg	Microgram
mg	milligram
$MgSO_4$	Magnesium Sulphate
MHz	Mega Hertz
µL	Microlitre
mL	Millilitre
mmol	Millimoles
MMV	Medicines for Malaria Venture
MeOH	Methanol
mol	mole
m.p	melting point
MRSA	Methicillin Resistant Staphylococcus Aureus
Na	Sodium
n	Integer
$NaHCO_3$	Sodium Hydrogen Carbonate
NaOAc	Sodium Acetate
NaOEt	Sodium Ethoxide
NaOH	Sodium Hydroxide
NCS	*N*-chlorosuccinimide
Na_2SO_4	Sodium Sulphate
ND	Not Determined
NH_4OH	Ammonium Hydroxide
nM	nanomolar
NMDA	*N*-methyl-D-aspartic acid
NMR	Nuclear Magnetic Resonance
NPP	New Permeability Pathway
o	ortho

O/N	Overnight
P450	Cytochrome P450
p	para
P.	*Plasmodium*
P1	Purine Transporter
P2	Aminopurine Transporter
PAH	Poly Aromatic Hydrocarbon
PC	Phosphatidylcholine
PCP	*Pneumocystis carinii* pneumonia
*Pf*crt	*Plasmodium Falciparum* Chloroquine Resistant Transporter
pKa	Acid Dissociation Constant
PMD	Pentamidine
ppm	parts per million
R	Room Temperature
RNA	Ribonucleic Acid
SAR	Structure Activity Relationship
SD	Standard Deviation
SDS	Sodium dodecyl sulfate
T	*Trypanosome*
TEA	Triethylamine
TEAB	Triethylammonium bicarbonate
Tert	Tertiary
TFA	Trifluoroacetic Acid
$TiCl_4$	Titanium Tetrachloride
TLC	Thin Layer Chromatography
TMS	Trimethylsilane
tRNA	Transfer Ribonucleic Acid
UV	Ultra Violet
WHO	World Health Organisation
Zn	Zinc

Contents

CHAPTER I

Vector Borne Infectious Disease

1.0 An Introduction to Vector Borne Infectious Disease

Cardiovascular disease is the foremost cause of fatality as shown in Figure 1, with infectious and parasitic disease being a close second.[1] However, this balance is dependent on location. The Western world is mainly affected by degenerative diseases where the structure or function of the affected tissue deteriorates, mainly due to age and/or lifestyle. In stark contrast, the developing world is held hostage to a multitude of vector-borne infectious diseases that are rampant in certain areas. Of these diseases African trypanosomiasis, leishmaniasis, malaria, dengue and dengue haemorrhagic fever are among the most prevalent, affecting the Western world with travel and tourism. Many of these diseases are the oldest in the world for instance malaria, a disease that prehistoric man is believed to have suffered. The level of malaria infection is now endemic in Sub Saharan Africa and South East Asia restricting economic growth and development.[2]

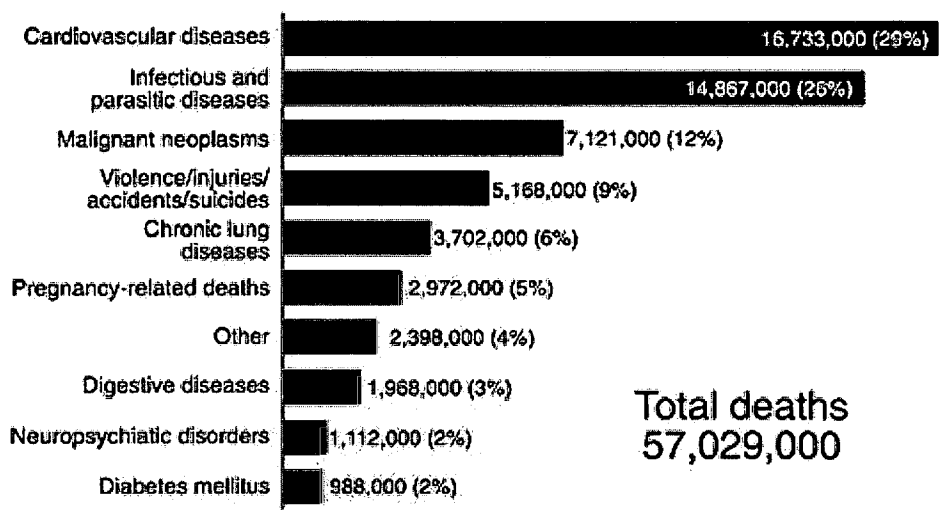


Figure 1. Leading causes of death worldwide 2002. Adapted from the National Institute of Allergy and Infectious Diseases.[1]

Assessing malaria specifically, prevention and control programmes have had some success. In 1930, a malaria epidemic in Brazil was eradicated due to the fast action of the Brazilian government and the Rockefeller Foundation. In addition to this, in 1943 *Anopheles gambiae* spread from Central Africa to Egypt, the Egyptian Government together with the Rockefeller Foundation again eradicated the disease in the affected Egyptian terratorries.[3]

During the Vietnam War and World War II, many resources were ploughed into the development of novel antimalarials leading to the development of mefloquine (America), halofantrine (America) and proguanil (Britain).[4] However, the collapse of the Empire and a reliance on chloroquine (CQ) led to the cessation of clinical research, drug development, and prevention and control measures. Even today only moderate resources from the pharmaceutical industry are placed into the development of novel, cheap therapies for the treatment of these diseases. The treatment of infectious parasitic disease is a neglected and underdeveloped area, for example Pentamidine (PMD) is still in clinical use today for the treatment of trypanosomiasis some 70 years after its introduction despite associated toxicity, and CQ for malaria, even though in most parts of the world CQ resistant *P. falciparum* malaria is now widespread.

Our interests lie within the development of novel chemotherapeutics for the treatment of malaria, thus accordingly the parasite lifecycle will be introduced to gain a better understanding of the processes we aim to target chemotherapeutically.

1.1 Malaria Epidemiology

1.1.1 Introduction

Originating in Africa, malaria accompanied human migration to India, South East Asia and the Mediterranean shores, due to the trade of slaves and goods. Once occurring widely in temperate areas, including Western Europe and the United States, malaria receded with economic development and public health measures.[5,6] Malaria is a major public health issue affecting more than a third of the world's population (approx 40%).[2] Every year, more than 500 million people become severely ill with malaria, with one billion people estimated to carry parasites at any one time. Pregnant women and children are the most vulnerable with the parasite killing one child every 30 seconds in Africa alone.[2,7]

In spite of global economic development, more people die from malaria today than 40 years ago,[6] causing much devastation and a staggering amount of chronic ill health that not only impedes the economic development of these countries already stricken by poverty, but also affecting fertility, population growth, worker productivity, and premature mortality.[8] The burden

of malaria can be observed clearly by certain genetic polymorphisms, such as sickle cell trait, selected due to the protective effect against malaria when inherited from one parent, though the same allele inherited from both parents is fatal.[9]

Malaria was reduced considerably in the 1950s due to the effectiveness of the World Health Organisation (WHO) prevention and control programme using drugs such as CQ together with 'residual' insecticides. However, drug resistance in the parasite and insecticide resistance in the vector exacerbated the situation, resulting in an explosion of treatment failures leading to the breakdown of the programme and the endemic situation present today. In addition, some of the newer antimalarials developed as alternative therapies have severe side effects and in most cases are more expensive than older treatments such as CQ, thus despite parasite resistance and treatment failures CQ is still the most widely used drug due to cost and availability.[10]

1.1.2 *Plasmodium* Life Cycle

The life cycle of malaria is complex and multifaceted since there is an asexual phase within the intermediate host and a sexual cycle in the mosquito.[11] Malaria is a vector-borne infectious disease of protozoan parasites of the genus *Plasmodium* transmitted by the female Anopheles mosquito. There are four species of *Plasmodium* which cause malaria in humans, *P.malariae*, *P.ovale*, *P. vivax* and *P. falciparum*, the latter two forms being the most prevalent. Due to the rapid rate of parasitic reproduction and an ability to sequester within vital organs such as the brain, *P. falciparum* causes the most severe clinical form.

Malaria transmission begins with the requirement for a mosquito to mature her eggs *via* a blood meal attained by a bite. Within her saliva she holds primitive malarial parasites named sporozoites that are injected *via* the salivary glands before feeding, thereby resulting in their release into the bloodstream where the parasite lives luxuriously, extracting nutrients and waiting to invade the CNS and other organs of their mammalian host, as outlined in Figure 2.

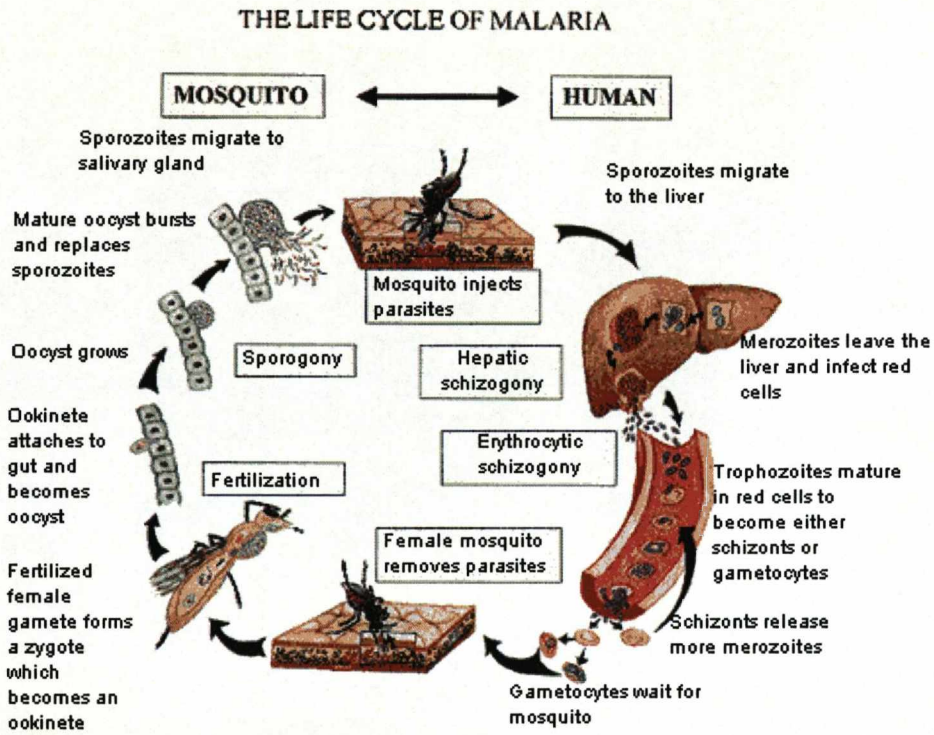


Figure 2. The parasite life cycle[12]

Once within the intermediate host bloodstream, sporozoites circulate for a short time, settling in the liver where pre-erythrocytic schizogony occurs when the parasites enter the parenchymal cells and multiply. After incubation periods of up to twelve days there can be merozoites (young parasites) in the order of thousands within one liver cell. The parasites exist within a cell in two forms consequential of sexual and asexual cycles. The sexual cycle creates male and female gametocytes which circulate in the blood and are taken by the mosquito when obtaining a blood meal. These gametocytes fuse forming oocysts in the stomach lining of the mosquito which develop over a few days. Within them are large colonies of sporozoites which move to the salivary gland ready to be injected into man. In the asexual cycle the budding parasites form schizonts which contain many merozoites. The cell undergoes lysis causing the release of more than 10,000 free merozoites armed to parasitize red blood cells thus completing the cycle. In the case of *P. malariae*, *P. vivax*, and *P. ovale* all stages of development prior to the liver cycle can be observed in the blood. For these strains the liver cycle continues though parasites are eliminated with a course of primaquine.[13] However, *P. falciparum* does not have a continuing liver cycle and only ring forms and gametocytes are present in peripheral blood. The developing

forms adhere to the blood vessels of large organs such as the brain, restricting blood flow leading to cerebral malaria.[2,5,12,14]

1.2 Antimalarial Chemotherapy

1.2.1 Introduction

Approaches to the control of malaria must be multifaceted in order to attain success; with vaccine development, health education, primary health care, chemotherapy and vector control working together alongside socio-economic improvements. Where malaria elimination programs have been successful, such as those implemented in the US and Europe, vector control was an essential program component[5] and is the course of action taken by various bodies funding projects for the development of novel, safe insecticides. Our interests are within the development of novel antimalarial chemotherapies and thus the history of antimalarial drugs will be briefly discussed.

There are many avenues of pharmacological intervention within the malarial parasite and the development of a therapeutic arsenal commenced with study of the parasite cell structure as illustrated in Figure 3. Furthermore, the recent elucidation of the malaria parasite genome sequence has provided a foundation for future studies of this organism and is being exploited in the search for new drugs and vaccines to fight the parasite.[15-20]

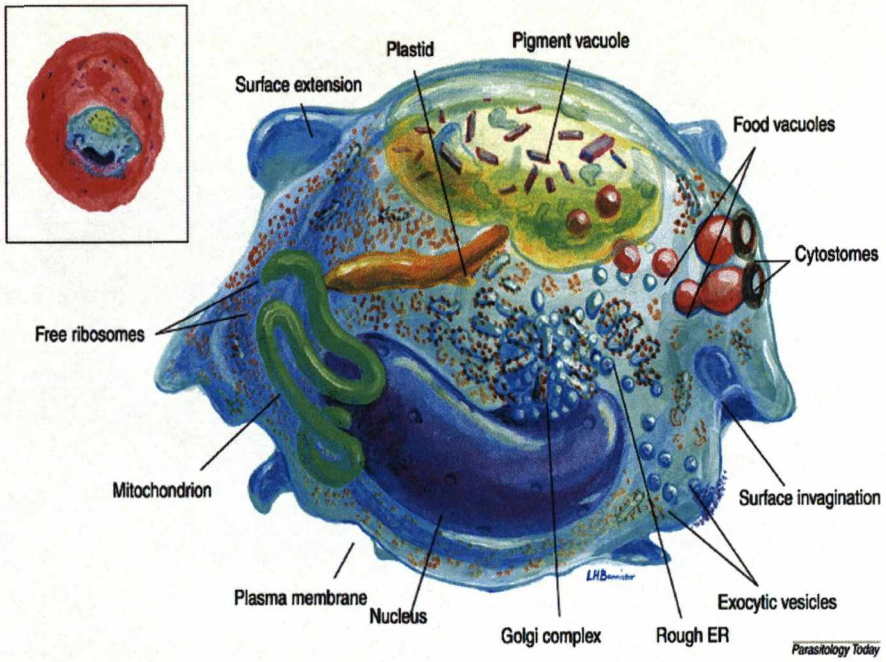


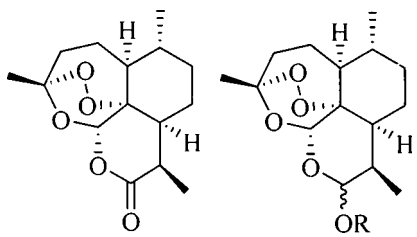
Figure 3. Structure of a parasitised red blood cell.

Within the parasitised host cell, haemoglobin is degraded for the attainment of amino acids, used for parasite growth and development. The parasite cannot survive without digestion of haemoglobin since it is unable to achieve the biosynthesis of many essential amino acids. This process occurs in the food vacuole where haemoglobin is degraded to heme and globin, the latter being used for amino acid synthesis. Heme is however toxic to the parasite and is detoxified by oxidation to hematin with subsequent crystallisation within the pigment vacuole. Aside from this process of haemoglobin degradation, it is clear from Figure 3 that there are many potential targets of bio-chemical intervention. Furthermore, since protozoa are unicellular eukaryotes, knowledge of structures such as the nucleus, rough endoplasmic reticulum and mitochondrion are well established.

The main groups of antimalarial drug therapies will now be discussed in addition to their mechanism of action.

1.2.2 Endoperoxides

The development of the endoperoxide antimalarial subclassclass is owed to the isolation of artemisinin **1**, a sesqueterpene lactone and the active ingredient within the Chinese herb 'qinghao' (*Artemisia annua*) used traditionally for treating fever.



1

2	R=H	Dihydroartemisinin
3	R=Me	Artemether
4	R=Et	Arteether
5	$R=CO(CH_2)_2CO_2H$	Artesunate

Figure 4. Artemisinin and its semi-synthetic derivatives

The development of semi-synthetic derivatives of artemisinin generated a water soluble derivative, artesunate **5**, and the analogues, artemether **3** and arteether **4**, all having superior activity and absorption to artemisinin. All derivatives are metabolised to the active agent dihydroartemisinin *in vivo* acting against gametocytes, the sexual stages of the parasite that infect mosquitoes, thereby preventing transmission of parasites in subsequent blood meals.

Artemisinin although poorly soluble is fast acting, effective for both the treatment of acute *vivax* and *falciparum* malaria. The artemisinins do not have any effect on liver hypnozoites and are not useful for chemoprophylaxis. Artemesinin can be administered orally, intramuscularly or by suppository; artemether orally or intramuscularly; and artesunate intramuscularly or intravenously. Artemisinin derivatives are extensively used against malaria; however cost, supply, short half-life and high recrudescence remain issues with this class of drug.[21,22] Thus they are mainly used in combinations with lumefantrine or mefloquine in order to avoid the coexistence of multiple parasite stages and increase treatment compliance, an approach adopted in most affected regions based upon the recommendation of the WHO.[23,24]

1.2.2.1 Mechanism of Action

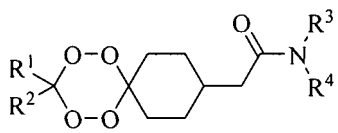
Many studies have been undertaken to elucidate the mechanism of action of artemisinin.[25-31] The mechanism by which these compounds act is poorly understood but is believed to involve iron-catalysed decomposition to form free radicals able to modify key biological targets.[4,31-33] Endoperoxides are known chemically to breakdown in the presence of iron to generate free radicals,[26,28,34,35] a factor that can explain the selective toxicity of artemisinin to parasites, since the malaria parasite is rich in heme-iron due to the breakdown of haemoglobin. Furthermore artemisinin and derivatives are inactive against parasite forms that lack hemozoin such as certain strains of *P. berghei*[36] and the related parasite *Babesia microti.*[4,37]

The endoperoxide bridge has been shown to be essential for activity.[30,38] For instance Brossi and co-workers synthesised and evaluated deoxy and peroxy derivatives of artemisinin. The deoxy analogues showed poor activity when compared to their peroxy counterparts, clearly demonstrating the requirement of the endoperoxide bridge for antimalarial activity.[39]

The reactivity of 1,2,4-trioxane molecules structurally related to artemisinin assessed with the heme model manganese(II) tetraphenylporphyrin, led to the observation that pharmacologically active drugs form covalent adducts by the addition of a drug-derived radical onto the porphyrin macrocycle, whereas no reaction is obtained with compounds lacking activity, suggesting that alkylation is likely to be one of the key factors of the pharmacological activity of endoperoxide-based antimalarial drugs.[40] It has also been reported that *in vitro* dihydroartemisinin and artemisinin react covalently with several malaria specific proteins for instance, the translationally controlled tumour protein (TCTP) in the presence of hematin.[41] Furthermore, it has been shown that artemisinins, but not quinine or CQ, inhibit the plasmodial protein sarco endoplasmic reticulcum Ca^{2+}-ATPase (SERCA) orthologue (PfATP6) of *P. falciparum.*[42]

1.2.2.2 Synthetic Endoperoxides

Several novel peroxide antimalarials have been synthesised based on the knowledge that the endoperoxide-bridge is crucial for activity leading to the development of among others, synthetic tetraoxanes and trioxalanes.[32,43-47] Amewu and co-workers assessed a range of orally active dispiro 1,2,4,5-tetraoxane **6** with activity superior to artemisinin.[48,49]

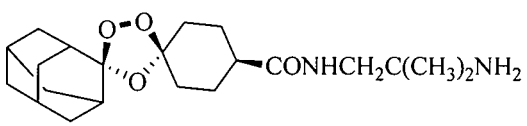


R_1, R_2 = cyclic ketones
R_3, R_4 = amines, amino acids

6

Figure 5. Novel tetraoxanes

In addition, trioxolane **7** was selected for Phase I clinical trials in humans since it has been shown to possess potent antimalarial activity against both *P. falciparum* and *P. berghei* in addition to comprising a good toxicology profile.[44] Hybrids such as **8** that attempt to combine two pharmacophores have been developed by Meunier and co-workers.[50] Although potent many of these "trioxaquines" have been prepared as mixtures of enantiomers and diasteriomers.



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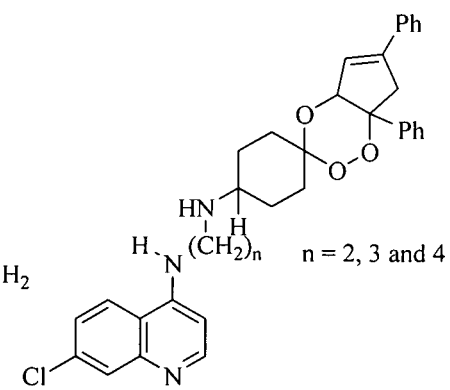


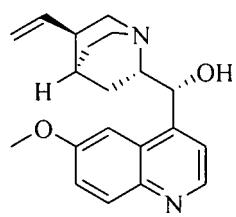
n = 2, 3 and 4

8

1.2.3 Quinoline Containing Antimalarials

1.2.3.1 Aminoquinolines

Natural products quinine **9** and isomers quinidine **10**, cinchonidine **11** and cinchonine **12** are alkaloids found in the bark of the cinchona tree that were used by native South Americans for centuries for the treatment of malaria (quinine is the most active ingredient). In 1908, the structure of quinine was theorised and later confirmed in 1944. Subsequent structure activity relationships confirmed that the quinoline nucleus is essential for activity, an understanding that led to the development and assessment of various synthetic derivatives creating the 4-aminoquinoline (4AMQ) antimalarials.



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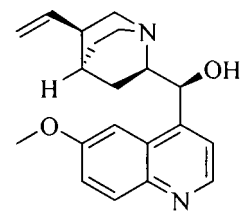

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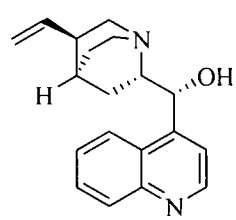

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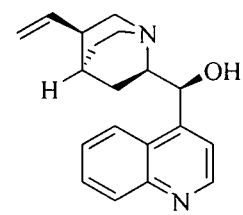

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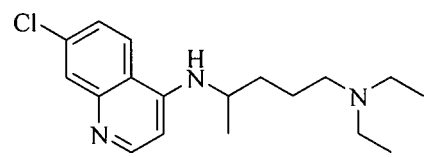

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Figure 6. Structures of quinine **9**, quinidine **10**, cinchonidine **11** cinchonine **12** and chloroquine **13**

The most significant example of Ehrlich's 'magic bullet'[51] within the field of antimalarial drug design is undoubtedly CQ **13**. CQ is one of the most important synthetic antimalarial chemotherapeutic agents in history since it is well tolerated, cheap and potent against all forms of *plasmodium*. CQ is a potent schizonticide that does not inhibit the liver cycle but has been used for both treatment and prophylaxis of malaria. Without doubt the demise of CQ through parasite resistance produced the biggest blow to the control and eradication of malaria. The molecular basis for the development of CQ resistance has been under much debate, with studies conducted by Bray and co-workers suggesting that modifications in the food vacuole lead to the decreased uptake of CQ by CQ resistant parasites, rather than enhanced cellular exit of preaccumulated drug.[52] In addition, they found that the initial rate of CQ accumulation by resistant parasites is increased by the calcium channel blocker Verapamil,[53] although this cannot be used for the clinical treatment of malaria.

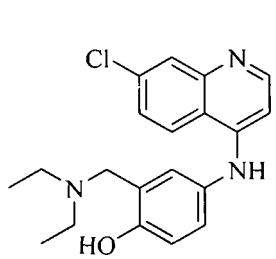
1.2.3.2 Mechanism of Action

CQ causes many biomolecular alterations, including the rapid degeneration of ribosomes, inhibition of protein synthesis and dissimilation of ribosomal RNA, when used against *Bacillus megaterium*.[54] As yet a precise mechanism of parasite death by CQ is not completely understood. It is, however, generally accepted that inhibition of hemozoin formation and FPIX-CQ interactions are a crucial part of their antimalarial efficacy. In the presence of free hematin, CQ and quinidine associate with the hematin monomer.[55] Furthermore, inhibitors of haemoglobin digestion antagonise CQ activity.[56] It is believed that the association of the quinoline nucleus with hematin restricts the growing hemozoin crystal preventing further sequestration of additional heme that then accumulates to levels that kill the parasite.[55] In addition, a correlation was observed between the hematin binding constant of these compounds and their ability to inhibit hematin crystallisation, suggesting that these compounds mediate their activity through binding to hematin.[57]

CQ is a dibasic drug which diffuses down the pH gradient to accumulate about 10,000-fold in the acidic food vacuole of the parasite (pH 5.4-5.5[58]). It has been shown that CQ uptake is due to binding of CQ to hematin rather than active uptake,[52] or by exchange for protons, by the

plasmodial Na^+/H^+ exchanger, and is therefore independent of NHE (Na^+/H^+ exchanger) activity.[59] Furthermore, 4AMQs related to CQ have been shown by SAR to require electron withdrawing groups at the 7-position of the quinoline ring for activity against hematin polymerisation and parasite growth, with chlorine giving the most potent activity.[60]

There are a vast range of 4-aminoquinoline and quinoline containing antimalarials developed as analogues of CQ, possibly the most significant of which are amodiaquine (4-aminoquinoline) **14** and mefloquine (quinoline methanol) **15**.



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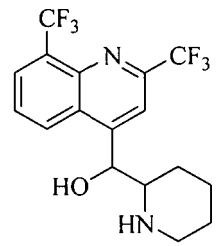
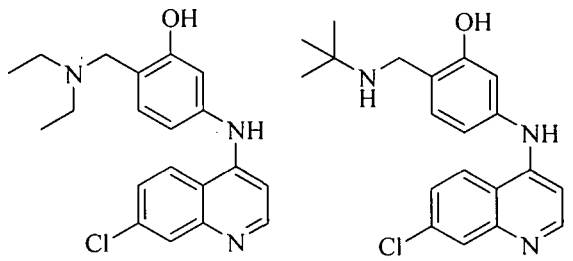


15

Amodiaquine has been part of the chemotherapeutic armoury against malaria, as an alternative to CQ since the 1940s. Generated through mass screening programmes in the U.S. at the end of World War II it was used for prophylaxis in 1963 due to problems in Vietnam with CQ resistant malaria. However, adverse side effects linked to metabolic activation *in vivo* have limited its clinical use. Furthermore, cross-resistance between amodiaquine and CQ has been reported.[4,61,62]

1.2.3.3 New Strategies

Isoquine **16a** is a 4-aminoquinoline antimalarial developed by O'Neill and co-workers as an alternative to amodiaquine. By interchanging the 3' hydroxyl and the 4' Mannich side-chain function of amodiaquine they produced a compound with potent *in vivo* and *in vitro* activity. Furthermore, isoquine does not undergo bioactivation *in vivo* thus having potential as a safer alternative to amodiaquine.[63] A drawback with **16a** was extensive first pass metabolism of the diethylamino side-chain. This was solved by replacement of this moiety with an *N-tert*-butyl functional group giving analogue **16b**. *N-tert*-butyl isoquine (**16b**) has just entered Phase I clinical trials in humans in a project sponsored by GSK and MMV.



16a **16b**

1.2.4 Quinolinemethanols

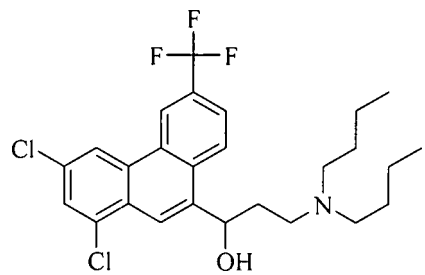
The quinoline methanol subclassclass are synthetic derivatives of quinine developed alongside the aminoquinoline antimalarials. Mefloquine (Lariam®) **15** is a quinolinemethanol that exhibits potent antimalarial activity for both the treatment and prophylaxis of malaria, though resistance was reported shortly after introduction.[64] The quinolinemethanols are understood to share their mechanism of action with CQ since it has been shown that CQ competitively antagonises mefloquine accumulation and mefloquine competitively antagonises CQ accumulation.[65] Furthermore blocking heme release with a protease inhibitor such as RO40-4388, an inhibitor of plasmepsin I, reduced the incorporation of radiolabelled CQ and quinine into malarial pigment by 95%, while causing a 70% reduction in the incorporation of radiolabelled mefloquine.[56] In addition, the cysteine protease inhibitor E64 reduced the incorporation of CQ and quinine into malarial pigment by 60 and 40% respectively, confirmed later by Sullivan and co-workers finding that inhibition was antagonistic to mefloquine action as it is to CQ action. This suggests a common mechanism for quinoline antimalarial action dependent on drug interaction with both hematin and hematin polymer.[55] Although mefloquine interacts weakly with free FPIX it has been shown to inhibit FPIX polymerization *in vitro* with an efficiency similar to or somewhat less than that of CQ.[4,57]

Since Mefloquine exhibits potent activity *versus* CQ resistant parasites it has been widely used for prophylaxis and treatment of patients with CQ resistant malaria and is usually well tolerated though severe adverse effects have been reported, some of which have been reported as permanent.[66,67]

1.2.5 Quinoline Related Compounds

1.2.5.1 Phenanthrene Methanols

The phenanthrene methanol antimalarial drugs were developed as analogues of quinine as part of the Walter Reed Army Institute of research screening programme,[68] where the two fused rings of quinoline were replaced by other aromatic systems resulting in the use of a three ring phenanthrene system. The 9-phenanthrenemethanol halofantrine **17** (HF, Halfan®) showed the most potent activity[69] and has been used clinically with some success.[70,71] However, severe and potentially fatal side effects are associated with its use.[72,73] Furthermore, HF pharmacokinetics are variable due to poor bioavailability. Though absorption is enhanced by administration with fatty foods, this leads to unpredictable and often unacceptably high serum levels.[4] Resistance to HF is linked to a mutation in the *pfmdr-1* (*plasmodium falciparum* multi-drug resistant) gene as observed for related compounds quinine and mefloquine.[74]



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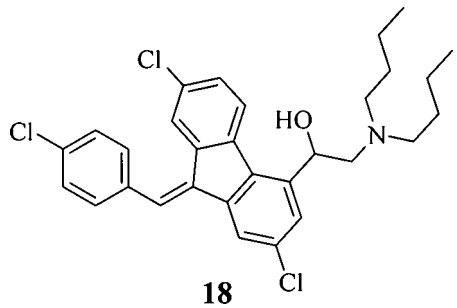
1.2.5.2 Lumefantrine

Lumefantrine **18** is a racemic fluorene methanol synthesised and assessed for antimalarial activity in Beijing.[75] It shares many properties with HF, including structural similarities in addition to poor and variable bioavailability resulting in a requirement for co-administration with fat.[76,77] A joint development between Novartis Pharma and the Academy of Military Medical Sciences (Beijing, China)[75] led to its successful use in combination therapy with artemether (Coartem®).[78,79]



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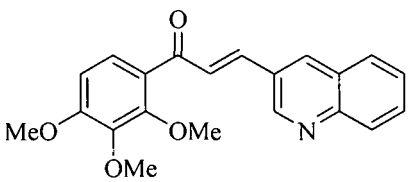
1.2.6 Protease Inhibitors

Protease inhibitors are used routinely as part of HIV-1 (human immuno deficiency virus) combination treatment regimens[80,81] and have displayed significant activity against malaria parasite growth.[82] Therefore protease inhibitors have applications within malaria treatment and prophylaxis in addition to the treatment of HIV/ malaria co-infection.

The intraerythrocytic degradation of haemoglobin is required for amino acids used for parasitic growth and development, believed to be an ordered process mediated by protease enzymes.[83,84] *Plasmodium* aspartic protease enzymes, termed plasmepsins, degrade host cell haemoglobin releasing amino acids and toxic monomeric heme and it is believed that plasmepsins I, II and IV are responsible for the initial cleavage.[85] The process is specific with Plasmepsin I being responsible for the initial cleavage of the haemoglobin tetramer at the hinge position, making a single cleavage between α-33Phe and 34Leu and since this takes place at the hinge position, the molecule unravels exposing other sites for proteolysis.[86] A second aspartic protease, Plasmepsin II, has also been identified and may have a role in the cleavage of denatured haemoglobin.[84] Falcipain, a cysteine protease of the papain family, is also implicated in the cleavage of peptides from the denatured haemoglobin.[84] There are four known falcipains (FP) labelled FP-1, FP-2, FP2' and FP3 with FP2 and FP3 being responsible for haemoglobin hydrolysis. Furthermore, it has been shown that alterations in the FP2 gene cause the accumulation of undegraded hemoglobin in trophozoites.[87-89] The globin fragments resulting from this process are presumably transported to the parasite cytosol where they are degraded further to release amino acids that are used by the parasite. As previously discussed, it is generally agreed that the remaining hematin

residue, which is potentially toxic, is removed *via* a polymerization process, degradation, or export.

Several protease inhibitors have been developed with chalcone **19**[90] and phenothiazine **20**[91] showing *in vitro* antimalarial activity, therefore becoming a promising class of antimalarials particularly applicable for combination therapies.



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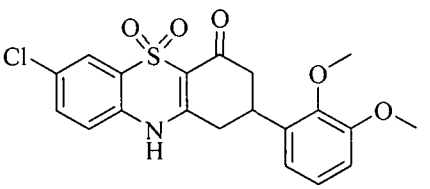

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1.2.7 Others

Antifolates – Knowledge of cell biology led to the development of the antifolates examples of which are pyrimethamine, proguanil, chloroproguanil, sulfadoxine and dapsone all acting on the folate pathway thereby preventing DNA synthesis as shown in Figure 7. Antimalarial antifolates have been central for prophylaxis and treatment of malaria. This drug family was discovered during World War II, since this time no novel antimalarial antifolates have been developed that have reached Phase I/II stages since little research has been done in this area.[92]

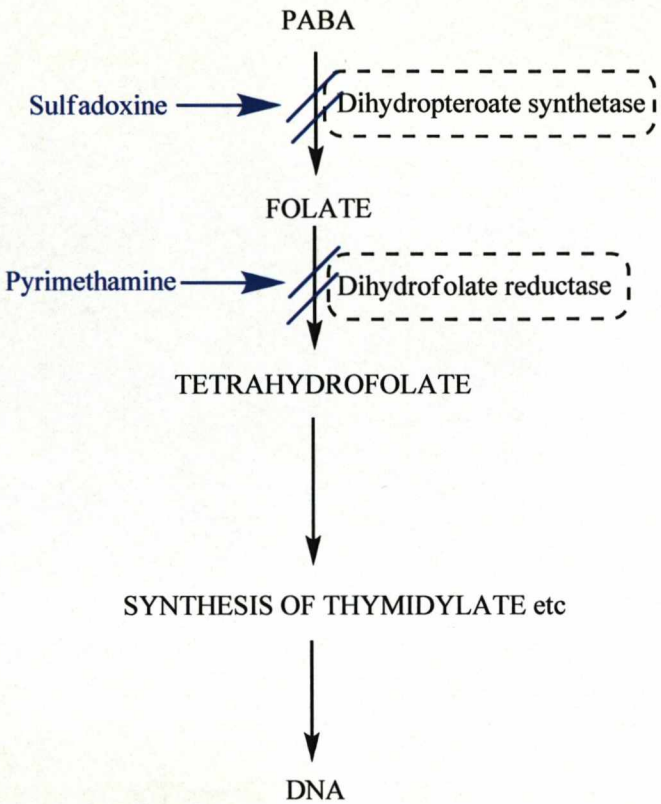
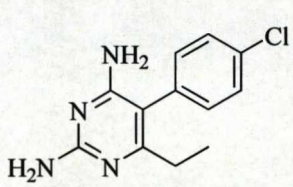


Figure 7. Folate biosynthetic pathway

The most clinically significant antifolate is the combination of pyrimethamine **21**, an inhibitor of dihydrofolate reductase, and sulpfadoxine **22**, a sulfonamide that interferes with the action of dihydropteroate synthetase. Both drugs act on the folate pathway thereby inhibiting the production and maintenance of new cells by impeding nuclear division at the time of schizont formation in erythrocytes and the liver.[93]



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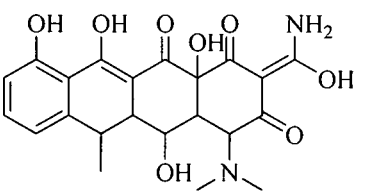
Fansidar® (pyrimethamine-sulfadoxine) acts synergistically, an advantage since this can be a method of negating parasite resistance mechanisms, although parasite resistance to this

combination is reported.[92,94] More recently, chloroproguanil has been developed in combination with dapsone as LapDap® by work supported by the WHO at the University *of* Liverpool with the cost of synthesis covered by GSK, WHO and the UK Department for International Development. The mechanism of action is synergystic by inhibition of the folate pathway at two points, in a similar manner to Fansidar®, since chloroproguanil targets dihydrofolate reductase and dapsone targets dihydropteroate synthetase similar to pyrimethamine and sulfadoxine respectively. Unforunately LapDap has been withdrawn from the market due to adverse reactions observed in a Phase III trial of LapDap and artesunate.

Tetracyclines – are a slow acting class of antimalarials whose mechanism of action is unclear[95] but believed to involve inhibition of protein synthesis, blocking expression of the apicoplast genome, resulting in the distribution of nonfunctional apicoplasts into daughter merozoites.[96,97] The most notable members of this class are the semi-synthetic tetracycline antibiotics doxycycline **23** and tetracycline **24** which are effective for the prophylaxis of malaria.[98,99]



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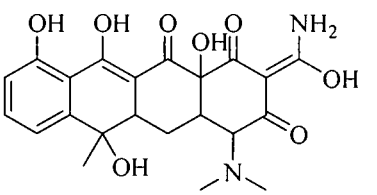

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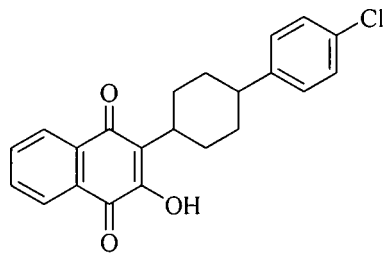
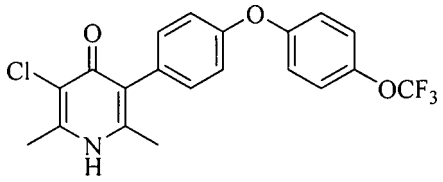
Napthoquinones have a broad spectrum of activity[100-102] and are believed to work by inhibition of parasite electron transport systems.[103,104] Atovaquone **25a** is a napthoquinione used in combination with proguanil as Malarone® for treatment and prophylaxis of malaria.[98,105] Recently GSK in collaboration with the MMV developed a novel class of pyridones with activity against atovaquone resistant parasites such as **25b**.



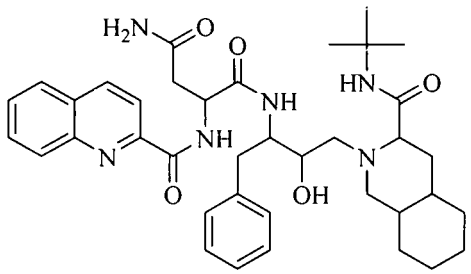
25a



25b

1.2.8 Drugs Used Against Other Diseases

As the biological knowledge of the malarial parasite grows there are various novel targets being exploited for the design of innovative chemotherapeutic strategies. Type 2 fatty acid biosynthesis,[106-108] plastid DNA replication and transcription,[95,109] glucose transport,[110] nutrient uptake,[111,112] and nucleotide biosynthesis[93,108,113] being examples of novel antimalarial targets. Furthermore the elucidation of the malarial genome and the discovery of its high AT (adenine and thymine nucleotides) content[15] termed super-AT islands,[114] have rendered compounds with an ability to bind to DNA potential drug templates.[114,115] A common approach to the design of novel agents for chemotherapy begins with the screening of drugs for other diseases thus there are many reports of this form of drug development within the literature such as folate antagonists,[116-119] natural products,[120-125] antibiotics,[126-128] antiretrovirals,[129,130] and iron chelators.[131-134] The most exciting of which is the study of HIV antiretroviral therapies since these protease inhibitors could have an added benefit in patients suffering from HIV and malaria. Although findings suggest that the effectiveness and tolerability of antiretrovirals in patients with malaria and HIV-1 co-infection may contribute to altered malaria disease outcomes.[135,136] However, a synergistic relationship between CQ and various HIV protease inhibitors is recognised[82,137-140] within both CQ resistant and -sensitive parasites with saquinavir **26**, ritonavir **27**, and lopinavir **28** being the most potent[137,139] both *in vivo* and *in vitro* although to date they have not been employed clinically.



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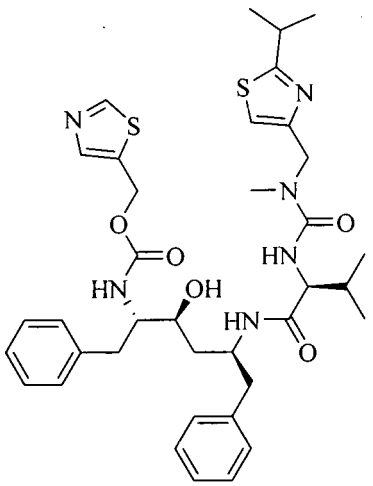

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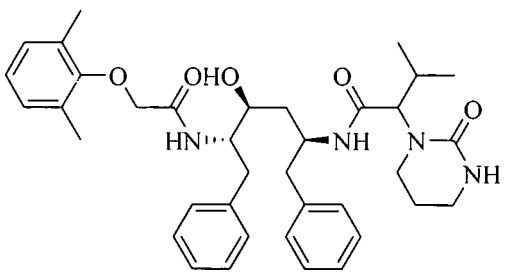

28

Of significance to us is the aromatic diamidine pentamidine **29** (PMD) used clinically for the treatment of the parasitic infections leishmaniasis and African trypanosomiasis (sleeping sickness) in addition to the fungal infection *Pneumocystis carinii* pneumonia (PCP) caused by *Pneumocystis jirovecii*,[141] an opportunistic infection in immunocompromised patients such as those receiving immunosuppressants but more frequently in HIV/AIDS patients. The antimalarial activity, possible mechanism of action and properties of PMD are discussed in detail within Chapter II since this is the seed of the research completed within this thesis.

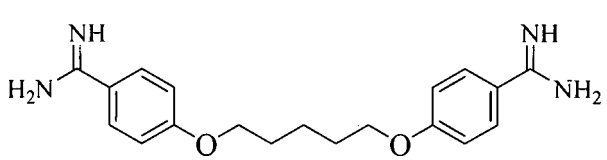

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Figure 8. Other antimalarials

CHAPTER II

PART I

Dicationic Antimalarial Chemotherapy

A review of the medicinal properties of dicationic compounds is important due to the nature of the work described in this thesis. The research undertaken is divided into two sections that discuss dicationic and *mono*-cationic antimalarial drug templates their development and antimalarial activity.

A separate numbering system will be used in this chapter for all Figures, Schemes and Compounds.

2.0 An Introduction to Dicationic Antimalarials

2.0.1 Ammonium Derivatives

Post plasmodial invasion, the parasitised erythrocyte becomes highly modified. Among these alterations, the phospholipid content of the infected erythrocyte increases approximately 500 fold, of which phosphatidylcholine (PC) becomes the major component at 40-45% of the total phospholipid content.[142,143] PC is required for membrane biogenesis, a process necessary for parasite growth and development. PC biogenesis does not occur in mature erythrocytes,[142] and it is synthesised *de novo via* the Kennedy pathway as shown in Figure 1[144] or by the decarboxylation of phosphatidylserine.[145,146] The Kennedy pathway is responsible for the majority of the phospholipid formed and is the most important pathway.[146]

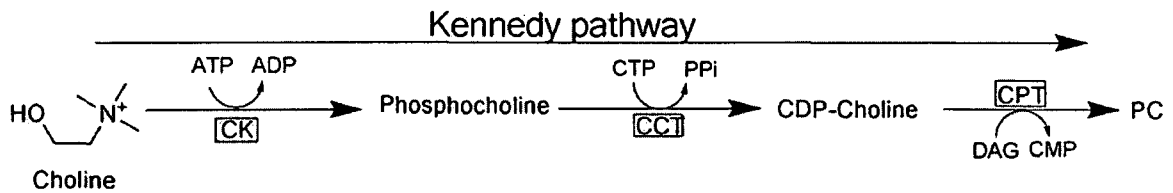
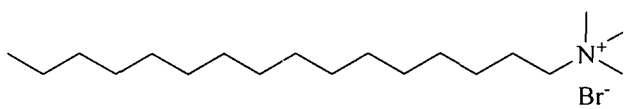


Figure 1. Kennedy pathway for PC biosynthesis and the structures of choline. Abbreviations; CK, choline kinase; CCT, choline phosphate cytidylyltransferase; CDP-Choline, cytidine diphosphate choline; CPT, choline phosphotransferase; DAG, diacylglycerol; PC, phosphatidylcholine.[144]

The first step of the *de novo* pathway involves incorporation of choline, followed by enzymatic steps with choline kinase (CK), choline phosphate cytidylyltransferase (CCT) and choline

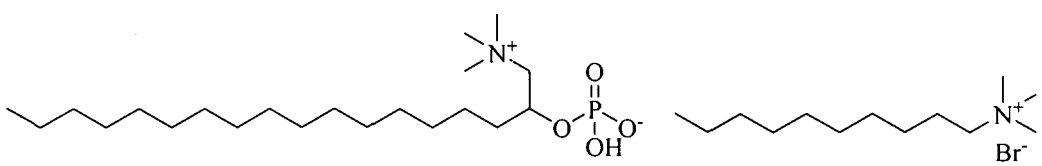
phosphotransferase (CPT) respectively to generate PC. CK is the first enzyme in the Kennedy pathway, the precise role of which with respect to parasite growth and survival is not fully understood, although it is believed to play a pivotal role in trapping the essential choline polar head group inside the malaria parasite.[144]

The incorporation of choline is essential for this pathway to occur, inhibition of choline uptake proving lethal to the parasite. Ammonium derivatives have been employed as analogues of choline, some of which exhibit potent antiprotozoal activity against *P. falciparum* inhibiting CQ sensitive and -resistant parasite forms.[144,147-150] Hexadecyltrimethylammonium bromide **1** is a potent inhibitor of *P. falciparum* growth *in vitro* and *P. yoelii in vivo* with trophozoite and schizont stage parasites being particularly sensitive to its effects.[144]



1

The mechanism of action of these ammonium salts is believed to involve inhibition of CK leading to a decrease in PC and parasite death.[144] Interestingly, the stage specificity of these molecules corresponds to the expression pattern of *P. falciparum*-CK correlating to a decrease in phosphocholine generation. Furthermore ammonium salts **2** and **3** are believed to inhibit CCK and CPT action respectively.[144]

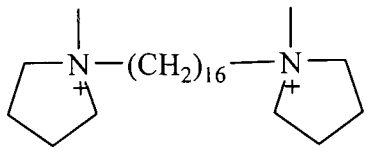


2 **3**

The evaluation of *mono*-cationic quaternary ammonium salts, led to the elucidation that antimalarial activity is related to the ability of quaternary ammoniums to mimic choline. Development of these compounds was essentially centred on the variation of carbon chain length.[144,147,150] Naturally, the next stage of development involved the evaluation of *bis*-quaternary ammoniums[150,151] allowing variation of the parent structure. The structural

requirements of *bis*-quaternary ammonium salts are similar to those of *mono*-quaternary ammonium salts i.e. polar head, steric hindrance and lipophilicity around the nitrogen function such as methyl, hydroxyethyl, ethyl and pyrrolidinium.[151]

G25 (**4**), is a product of this concept possessing potent antimalarial activity, inhibiting 50% parasite growth (IC_{50}) at a concentration of 0.65 nM.[152] In addition, G25 concentrates within infected erythrocytes to several hundred fold higher than the surrounding medium being1000-fold less toxic to mammalian cell lines, curing monkeys infected with *P. Falciparum* and *P. Cyanomolgi.*[153]



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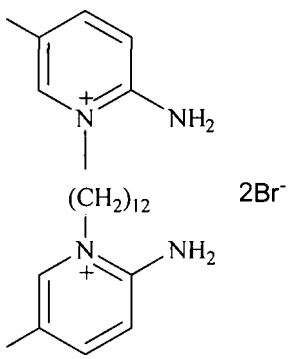
Naturally, additional analogues were generated in a bid to develop more potent compounds. Unfortunately, the poor oral bioavailability and toxicity associated with G25 became evident halting its development.[154] Using a similar strategy, *bis*-2-aminopyridinium salts such as **5** and **6** were generated displaying potent antimalarial activity with IC_{50}s of 0.5 nM although their oral activity is not reported.[152]



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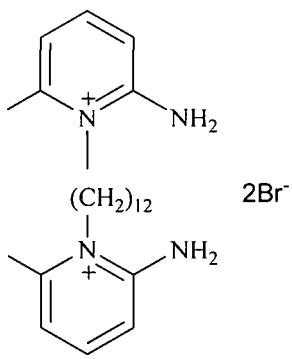

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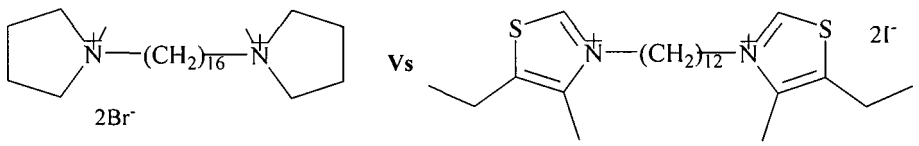
Bis-quaternary iodide salt **7** (T16) is a direct analogue of G25 showing potent antiplasmodial activity. Choline transport is the suggested mode of action for these compounds; however their precise mechanism of parasite inhibition as yet cannot be stated definitively. G25 and T16 inhibit choline uptake into *P. falciparum* and *Saccharomyces cerevisiae* infected cells, inhibiting their growth.[155,156] Furthermore, G25 inhibits both the *de novo* PC metabolic pathway (Kennedy pathway) and the synthesis of phosphatidylethanolamine from phosphatidylserine (alternative route) indicating that G25 specifically targets the pathways for synthesis of the two major phospholipids, PC and phosphatidylethanolamine to exert its antimalarial activity.[155]

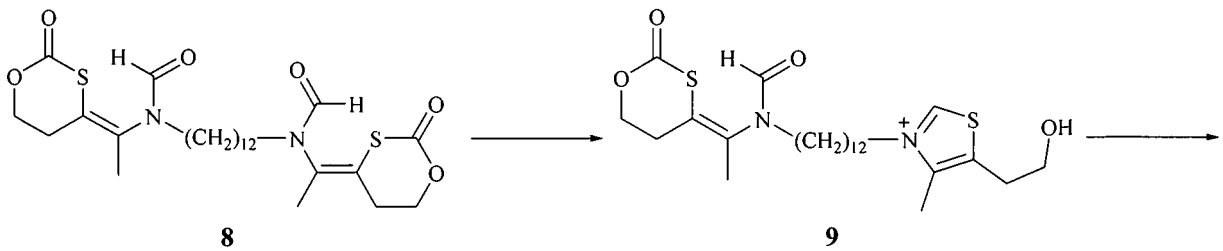


G25 **7**

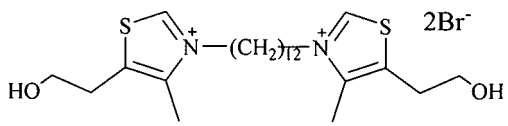
Interestingly, it appears that inhibition of phospholipid synthesis is not the sole mode of action by which these compounds work. T16 shows potent antimalarial activity and an ability to bind significantly to heme, an attribute which also correlates to the observed activity of this compound since accumulation assays reveal that this compound is readily concentrated several hundredfold into parasitised erythrocytes while approximately 80% of the drug was distributed within the parasite itself, 50% of which was located in the parasite food vacuoles. Furthermore, decreasing ferriprotoporphyrin IX (FPIX) concentration caused a marked decrease in the antimalarial activity of T16. In addition to these factors, it appears that this dication is a substrate of the induced permeability pathways present within malaria infected erythrocytes,[157] a process that will be discussed in more detail within section 2.5.3.

Further developments within this series led to the generation of the *bis*-thiazolium salt T3 **10** and its prodrug TE3 **8**. The non-ionic pro-moiety was developed in the hope that it would improve the bioavailability of the drug. Although in-depth metabolism studies on this prodrug do not appear to have been undertaken, an intermediate metabolite with a molecular weight of 577 is noted which corresponds to compound **9**. TE3 is rapidly converted *in vivo* to the active

compound, both prodrug and active moiety possessing potent oral activity with ED_{50}s of 5 and 13 mg/kg respectively *versus P. vinckei.*[158]

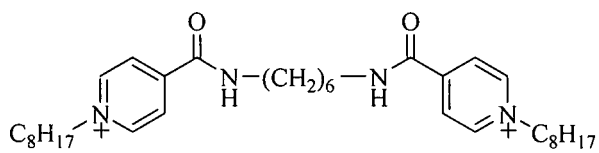


8 **9**

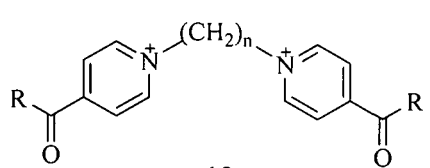


10

Motoshima and co-workers pyridinium series **12** is derived from antiplasmodial structure activity relationships developed previously for analogue **11**. From these studies they developed a range of dicationic analogues inexpensively from low cost materials in two synthetic steps.[159]

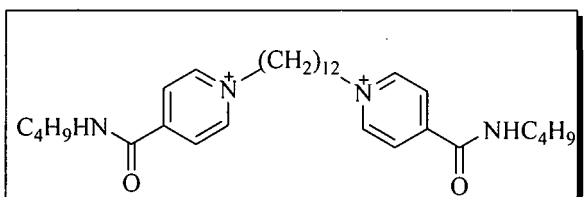


11

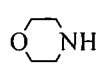


12

R = $C_mH_{2m+1}NH$ m = 4,6,8 n = 6,8,10,12



13 ED_{50} = 8.2 mg/kg

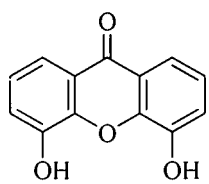


PhNH

Compounds bearing alkyl or phenyl groups were found to have enhanced antimalarial activity *in vitro* in comparison to other analogues tested. In addition, compound **13** (R= C_4H_9NH n=12) is a molecule with potent antiplasmodial activity *in vivo* (*P. berghei*-infected mice) with an ED_{50} of 8.2 mg kg^{-1}. Furthermore, the relative position of the amide bond and pyridinium ion had little effect on activity. In terms of *in vitro* activity, the lead compound **13** had enhanced activity compared to CQ however the *in vivo* activity of this analogue was reduced. The authors attribute this relationship to the high level of excretion resulting from the hydrophilicity imparted from their cationic character although some analogues showed enhanced activity *in vivo* compared to their *in vitro* activities and visa versa.[159] Studies into the mechanism of action of these analogues remain unpublished at present but based on those published for similar compounds presumably involve the inhibition of PC biogenesis and binding to FPIX.

2.0.2 Xanthones

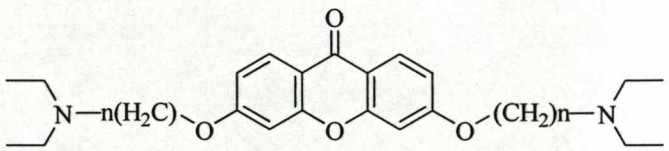
Hydroxyxanthones have been identified as novel antimalarial agents, believed to exert their activity within the parasite food vacuole targeting heme detoxification. 4,5-Dihydroxyxanthone **14** has been shown to subvert the detoxification of free heme by forming a hydrogen bonding interaction between the hydroxyl groups and propionate side chains of heme, in addition to π–π stacking between both aromatic systems forming a complex with the heme dimer. Furthermore the xanthone carbonyl moiety coordinates to the heme iron.[160]



14

Xanthones **15** and **16** are potent compounds designed as analogues of **14** with protonable side chains in an attempt to enhance the interaction of these compounds with the propionate functions of heme, while enhancing their accumulation into the acidic parasite food vacuole. The potency of the analogues increases with carbon chain length until carbon 6 after which activity begins to

diminish. Interestingly, the correlation between heme binding affinity and antimalarial potency is almost linear, suggesting that a high binding affinity to heme is a critical factor to their antimalarial potency.[161]



15; n = 6

IC_{50} = 0.07 μM *versus* all strains

D6; CQ sensitve, W2; multidrug resistant, F86; CQ resistant

16; n = 5

IC_{50} = 0.10 mM vs. D6
0.11 μM vs. W2
8.26 μM vs. F86

Compound **16**, was employed for confocal fluorescence microscopy studies to elucidate the subcellular location of this compound and was observed to be in the digestive vacuole, where it accumulates from 5 to approximately 33,000 μM within 1 hour of exposure to parasitised red cells through the mechanism of uptake described in Figure 2.[162]

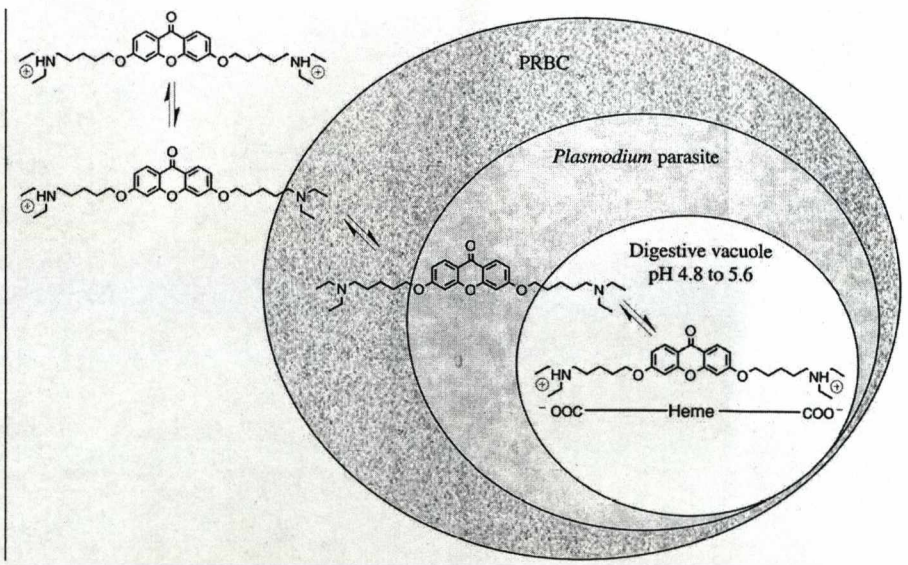


Figure 2. Proposed mechanism of uptake and accumulation in the acidic vacuole of *P. falciparum* -infected red blood cells.[162]

2.0.3 Summary

It is generally accepted that *bis*-ammonium compounds enter infected erythrocyte by new permeability pathways (NPP) after which they accumulate to high concentrations, a process required in order for them to exert their antimalarial effect.[163] They are not transported by the host erythrocyte choline carrier and penetration of the host cell compartment is solely through the NPP. They are however, carried by the parasite choline carrier therefore adding a level of selectivity over interactions with intracellular targets.[164] The precise anti-malarial mechanism of action of *bis*-ammonium compounds is not fully understood however and it has been shown that they inhibit the synthesis of phosphatidylcholine *de novo*, their precise point of inhibition varying between inhibition of choline uptake and/or the enzymes required in the pathway.[155] They have also been shown to be able to bind to FPIX, a process usually inferred within the accumulation of drugs within the parasite and antimalarial action.[157,163] Potent against multi drug resistant *P. falciparum* malaria these molecules are an exciting class of drug with TE3 scheduled to commence clinical trials, having the advantage that its mechanism of action is unexploited within present chemotherapeutic regimens.[165] It is quite likely that these compounds are dual molecules, exerting their antimalarial activity *via* two simultaneous toxic effects on intracellular intraerythrocytic parasites.[163]

Other functional groups with the ability to ionise readily at physiological pH are discussed below.

2.1 Diamidines

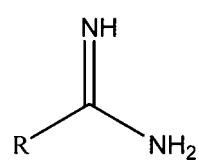
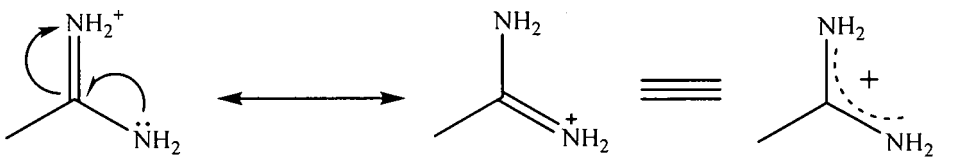


Figure 3. The amidine functional group

The amidine moiety consists of two nitrogen atoms bonded to a carbon centre as the N=C-N functional group shown in Figure 3. The pKa of the group is large (12.4[166]) due to the ability of

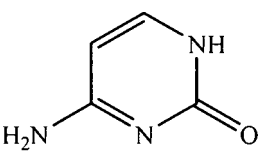
the nitrogen atoms to stabilise the positive charge by resonance forms **17** and **18**, the electronic distribution over the functional group represented by structure **19**.



17 **18** **19**

The existence of resonance form **18** gives rise to the possibility of restricted rotation about the C-N bond. Rotational barriers are expected to lie between those of single and double bonds being few tens of KJ mol^{-1} for single and few hundred kJ mol^{-1} for double bonds.[167]

The application of amidines has been demonstrated within synthetic chemistry as organocatalysts and nucleophiles[168-174] in addition to ligand based coordination chemistry.[175-177] Medicinally, diamidines find applications within the areas of cardiovascular,[178-181] antiviral,[182,183] antibacterial[184,185] and antitumor[186,187] drug templates in addition to being present within the DNA and RNA component, cytosine **20**.



20

Since the medicinal properties of diamidines form the basis of the research undertaken, they will be discussed separately under section 2.5.

2.2 Diguanidines

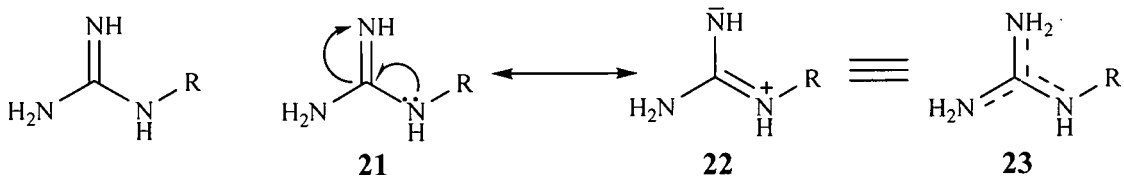
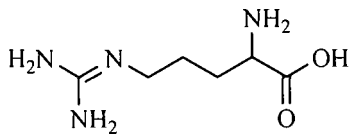


Figure 4. The Guanidine Moiety and Resonance Forms

The guanidine moiety consists of three nitrogen atoms bonded to a carbon centre as the N=C-N_2 functional group shown in Figure 4. Guanidines are more basic than amidines, the pKa of this group being 13.6.[188] The increased ability of the molecule to stabilise positive charge is derived from the formation of guanidinium ion **21**, the increase in electron density delocalised over three nitrogens, each having a third of a positive charge. The resonance stabilization imparted by the guanidine functionality and to a lesser extent the amidine, may be characterised in terms of properties commonly associated with "conventional" aromatic systems. Such properties include enhanced thermodynamic stability, delocalization of π-electrons through p-orbital overlap and energetic barriers to rotation of π-energy.[189] As a consequence, aqueous guanidine solutions are close in basicity to solutions of sodium hydroxide and are therefore readily protonated at physiological pH.[166]

Synthetically, guanidines are employed as catalysts, chiral auxiliaries and chiral bases within asymmetric synthesis.[190-197] Examples include the nitroaldol reaction,[198] Strecker reaction[199,200] and conjugate addition.[201,202] Interestingly, guanidines have found a role within the development of greener fuel systems aimed at replacing the chemical additives currently classified as dangerous by European legislation.[203] Juyal and Anand have developed guanidines, functioning as highly effective fuel stabilisers and dispersants that are non-toxic, non-polluting fuels.[203] Aside from their synthetic applications, interest surrounding the guanidine moiety centres around its biological importance. There are many guanidine and guanidinium-bearing natural products[204-209] such as those within marine systems,[210-214] the DNA nucleobases arginine **24** and

guanine **26** in addition to the skeletal muscle component creatine **25**, and endogenous compounds agmatine **27**, guanabenz **28** and amiloride **29**.



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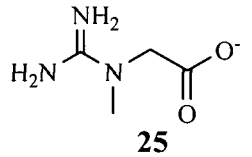

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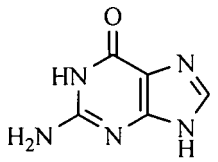

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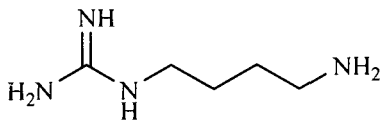

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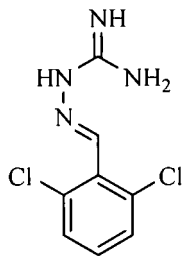

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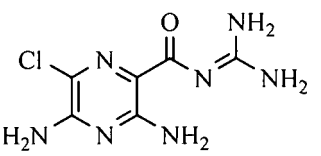
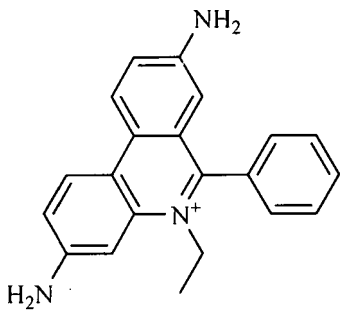
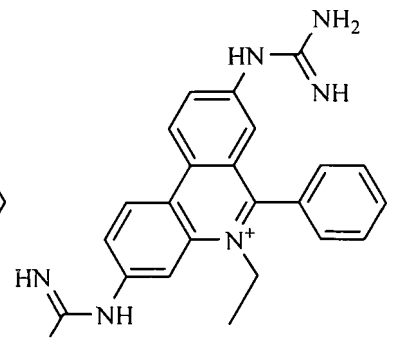


29

Within medicinal chemistry the guanidine moiety is frequently used as a DNA binding agent due to a high specificity for AT-rich DNA sequences. This property of the guanidine functionality can be further observed in the case of the *bis*-amine ethidium **30a**.



30a

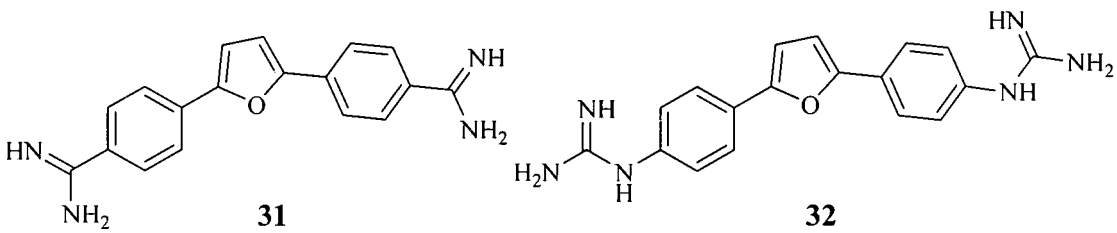


30b

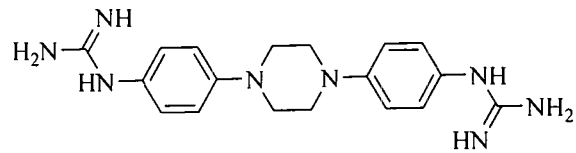
A monocationic intercalator of DNA and RNA, ethidium bromide intercalates into nucleic acid base pairs with little sequence specificity.[215-217] Interestingly, replacement of the amines for guanidines gives a ligand (**30b**) with selectivity for binding at AT-rich sequences in a non-intercalative manner, with a weak intercalatory interaction at GC sites. Furthermore, the binding strength of the *bis*-guanidine is much greater than that of ethidium.[217]

Guanidines and diguanidines boast many therapeutic properties which include; anticoagulant,[207] antifungal,[211-213,218,219] antiprotozoal,[212,220-224] cytotoxic,[211,213,225-228] antiviral,[226-230] inhibition of insulin release,[231] peptide mimetic,[232-235] neuroprotective,[236-240] protease inhibition[206] and have applications as drug delivery systems.[241] The antiplasmodial and antiprotozoal activity of guanidines have not been studied as extensively as diamidines, however several guanidine analogues have shown significant antitrypanosomal,[221,223,224] leishmanial[222] and antimalarial activity.[220,224]

Guanidines of furamidine **31** (DB75), a highly efficacious antiprotozoal compound currently in Phase II clinical trials, have been synthesised displaying potent activity *versus P. Falciparum* and *T. brucei. rhodesiense*.[220] Lead compound **32** displays enhanced activity *in vitro versus* plasmodial rather than trypanosomal protozoa. Furthermore, the activity of this compound is enhanced with respect to those of PMD and DB75 itself.



The antiprotozoal mechanism of action of diguanidines is unknown though they exhibit a high selectivity for the parasite[221] and are believed to be subject to transport mediated uptake.[242,243] Interestingly, diguanidines have been shown to possess potent antiplasmodial activity as shown in Figure 5, though this analogue is a poor inhibitor of hemozoin formation *in vitro* inferring a different mechanism of action.[244]

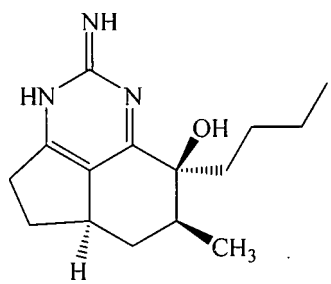


IC_{50} = 15.2 nM

Figure 5.

In terms of their antitrypanosomal activity, it is believed that binding to DNA at AT-rich sites is part of their mechanism of action, as guanidines with this form of activity have been shown to bind specifically at this region,[223,244] an interesting point when considering the AT richness of the malarial parasite genome.[15,115] However, it is unclear what role this binding interaction plays within the cessation of trypanosomal survival mechanisms since there is evidence that binding to DNA does not directly kill the parasite,[220] but rather leads to the inhibition of DNA dependent enzymes or inhibition of transcription by inducing alterations within the base pair structure.[245,246] Interestingly, the ability of guanidine **31** to bind to DNA was of a lower affinity than that of PMD and DB75, being 20-40% lower than the latter.[220]

Other guanidines with antimalarial activity include the tricyclic guanidine alkaloids **33** and **34**, identified from the marine sponge *Monanchora Unguifera*. A mixture of which was found to be active against *P. falciparum* with an IC_{50} value of 3.81 μg/mL.[212]



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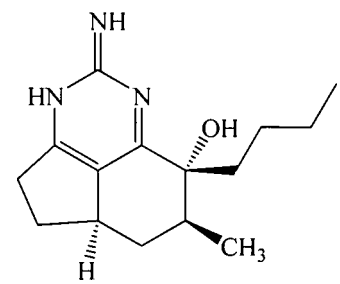


34

Diguanidines and *N*-alkyl diguanidines have been synthesised using the fluorene template showing potent *in vitro* activity against both *T. brucei rhodesiense* and *P. falciparum*.[224] The antimalarial activity of the most potent compounds *versus P. falciparum* is shown in Figure 6 alongside their associated IC_{50} values.

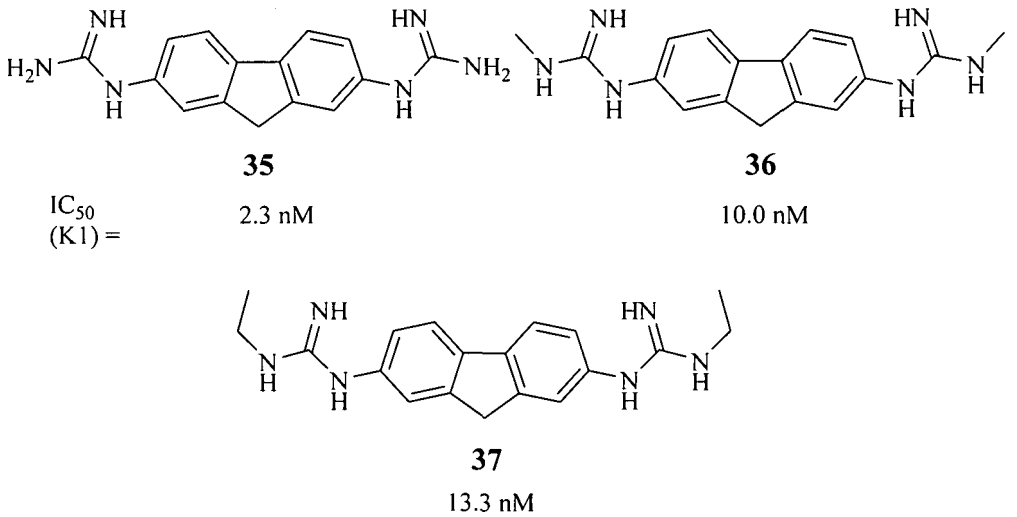


Figure 6. Fluorene Diguanidines

Clearly, addition of the *N*-alkyl group causes a reduction in antiplasmodial activity as observed for compounds **36** and **37** showing a decline in activity with increasing alkyl size; an isopropyl analogue gave an IC_{50} of 35.7 nM. Interestingly however, the *N*-methyl analogue showed high activity *in vivo* although this was only assessed in the African trypanosomiasis animal model. The antimalarial mode of action of these compounds was not studied though the ability of these compounds to bind to DNA at the minor groove was confirmed.

Finally, within our assessment of dications as antiplasmodial agents, the finding that *bis*-tertiary amines such as G25 experience enhancement in activity due to the nature of the dication was of importance. It has been shown that when the dication is a *bis*-amidine or *bis*-guanidine the activity of the compound is improved by approximately 2-fold against *P. falciparum* as shown schematically in Figure 7.[152]

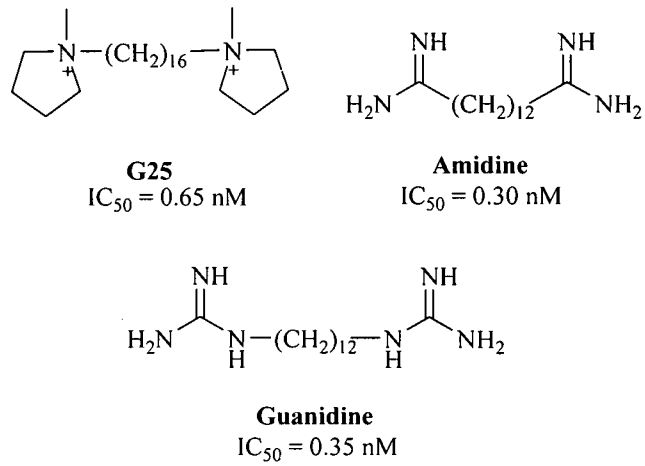


Figure 7. Effect of dication replacement on antiplasmodial activity

2.3 Reversed Diamidines

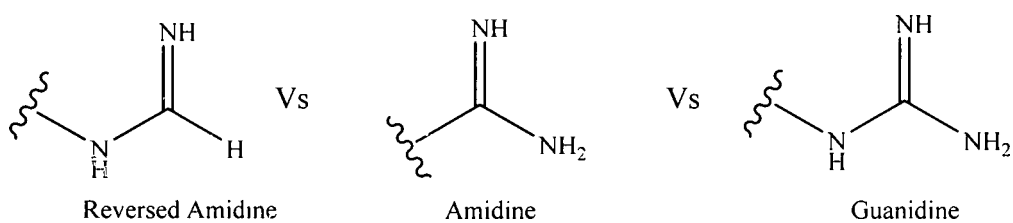
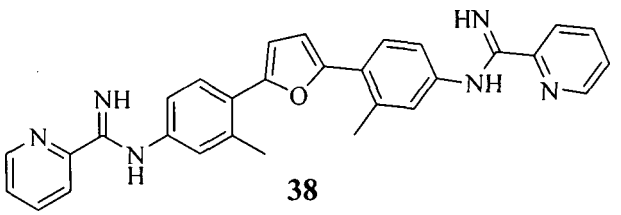


Figure 8. Structures of reversed amidine, amidine, and guanidine

The 'reversed amidine' moiety essentially comprises of an amidine functionality 'reversed' due to the bonding position to the desired drug template as shown in Figure 8. The concept of a 'reversed amidine' is derived from the requirement for structure based relationship studies into the ability of this moiety to interact with DNA.

The antiprotozoal activity of reversed-amidines have been studied, although less than diamidines, particularly for antiplasmodial investigations. The antitrypanosomal and antileishmanial activity of reversed diamidines have been assessed against their diguanidine counterparts finding that a reversed amidine series was most effective against *T. cruzi* and *L. donovani*.[222,247,248] In addition, Stephens and colleagues synthesised reversed amidine analogues

of 2,5-diarylfurans for studies into their antimicrobial activity finding that compound **38** was fungicidal.[219]

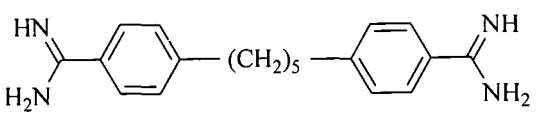


38

The mechanism of action of the reversed amidines is unknown, although DNA binding is believed to play a role. Reversed amidines have been shown to induce cellular effects such as altered morphology of nuclei, swelling of endoplasmic reticulum and Golgi structures in addition to damage of mitochondrial and kinetoplast structures[249] thus additional targets are believed to play a role.

Unfortunately, enhanced toxicity *in vivo* and poor oral bioavailability has limited the exploration of reversed amidine ligands. To date, a successful reversed amidine prodrug has not been generated.

Following the principles of drug design, our objective is the development of an agent toxic to the target whilst being non-toxic to host cells and host biological systems. Our interest in dicationic molecular systems will become evident, based on structural differences between *plasmodium* infected and non-infected cells. The *bis*-ionic systems chosen for development are the diamidine and diguanidine moieties based on their antiplasmodial properties. Naturally, our work commenced with the development of a structure activity relationship profile to gain information on the requirements of a drug template. We decided to use the aromatic diamidine PMD since its antimalarial activity is known *versus P. falciparum*, the most virulent malarial strain.[250]

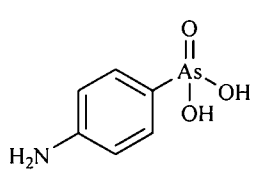


PMD

PMD is used clinically for the treatment of African trypanosomiasis, leishmaniasis and *Pneumocystis carinii* pneumonia. Although knowledge of the antimalarial activity of PMD is known, PMD has never been employed clinically for the treatment of malaria. Thus part of this chapter will discuss properties associated to trypanosomiasis, leishmaniasis and *P. carinii* pneumonia activity as knowledge of these systems are important to gain an insight into the properties of the compounds we are working with.

2.4 An Introduction to Diamidines and Their Development

In 1902, Thomas (Liverpool School *of* Tropical Medicine) demonstrated the trypanocidal activity of the synthetic compound *p*-aminophenylarsenic acid or atoxyl **39** in mice and rats infected with trypanosoiasis.[3] Bertheim, Hata and Ehrlich broadened the target organisms to include the causative agents of syphilis, the spirochaete bacterium *Treponema pallidum*. After the elucidation of the structure of atoxyl, the two chemists and microbiologist Hata commenced medicinal chemistry drug design as we know it today, by varying the atoxyl structure leading to the discovery of salvarsan in 1910.[251] The structure of salvarsan itself has caused much debate, the structure has however recently been confirmed as a mixture of compounds **40** and **41**.[252]



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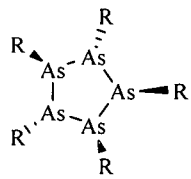

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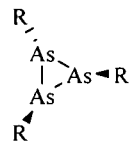

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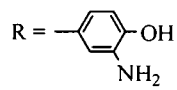
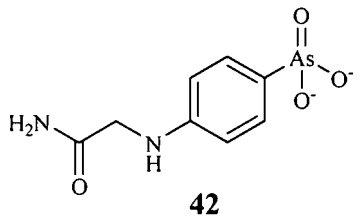


With the subsequent development of tryparsamide **42** at the Rockefeller institute for the treatment of trypanosomiasis and neurosyphilis, it seems the scientific community at this time were keen on the development of arsenides. In addition, tryparsamide had the advantage that it could penetrate the blood brain barrier (BBB) and thus was active during late stage sleeping sickness, a state that is hard to blockade.



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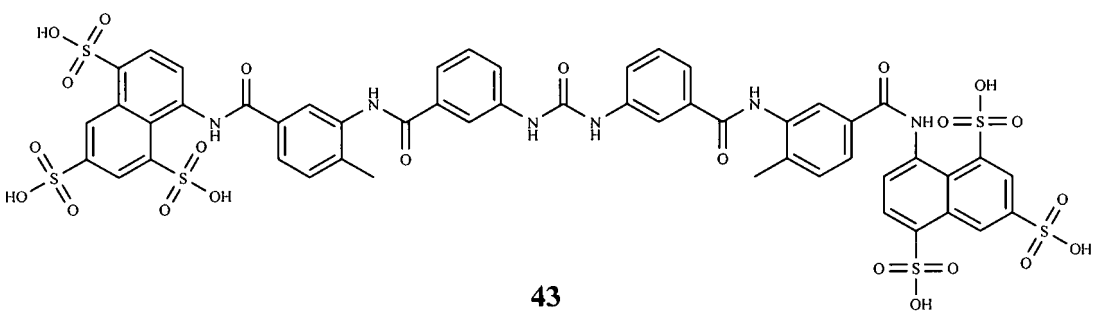
The first purely organic compound for the treatment of trypanosomiasis was Germanin **43** (or Suramin), highly effective for early stage disease but not late stage as it cannot penetrate the BBB.



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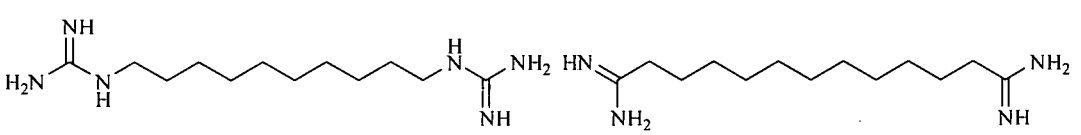
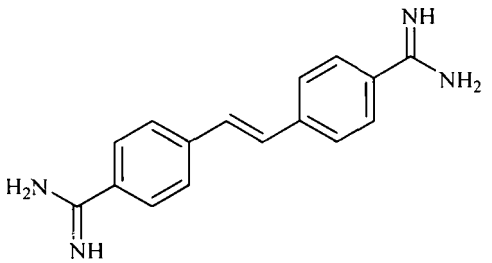
The toxicity of arsenides and the effectiveness of Germanin led to the use of diamidine and diguanidine moieties as analogues, studied for the treatment of trypanosomiasis, presumably commencing with the replacement of the carbonyl oxygen function of Germanin for a nitrogen moiety.

Lourie and Yorke first demonstrated the activity of dicationic compounds *versus* trypanosomes[253] by noting the curative properties of decamethylenediguanidine dihydrochloride **44** later finding that diamidinoalkanes were more active than diguanidines,[254] the most active being 11-diamidinoundecane dihydrochloride **45**.[255]



44 . 2HCl **45** . 2HCl

The work of Lourie and Yorke began the investigation of dicationic structures and analogues were synthesised by various groups,[3,255] such as Kirk *et al.* 4,4'-diamidinostilbene **46** and Adams *et al.* 4,4'-diamidinodiphenoxypentane **47** employed for the treatment of Mediterranean and Indian kala azar.[255] In 1942, Ashley studied the relationship between the chemical constitution and trypanocidal activity of a number of aromatic amidines[255] finding that the greatest activity was obtained by *bis*- rather than *mono*-amidines of the type **48** where X is an alkyl chain; the most active being when one or more of the CH_2 groups is replaced by O. Reviewing the literature, this is the earliest sighting of PMD, used extensively by the Belgians, British and French for the treatment of trypanosomiasis during the colonial period.[3]



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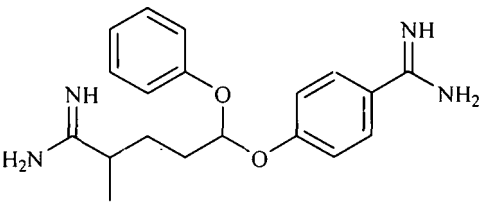

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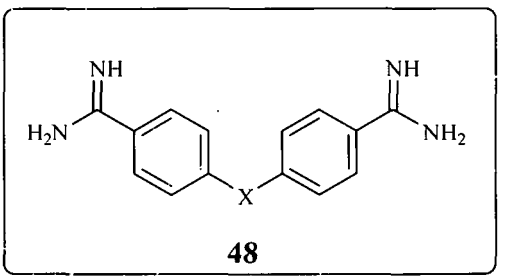

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2.5 The Medicinal Chemistry of Diamidines

2.5.1 Introduction

The amidine moiety is often used for staining or molecular tagging applications due to its strong fluorescence[256-258] and is a pharmacophore frequently employed within medicinal chemistry for its antiparasitic,[259-261] anticoagulant,[180,262,263] amoebicidal,[264-267] insecticidal/pesticidal,[268,269] antiviral,[270-274] antifungal,[275-278] antibacterial,[276,279-281] antihypertensive,[282] antitumor,[283-288] antiinflammatory[289,290] and analgesic properties.[290] The attributes of the amidine moiety are not restricted to biological systems since it is also used within organic synthesis for instance as a chelating ligand,[291-293] however it is their medicinal properties that will be discussed in detail commencing with the key compound pentamidine.

2.5.2 An Introduction to Pentamidine (PMD)

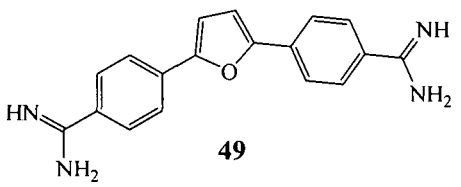
Pentamidine is an odourless, white, hygroscopic aromatic diamidine supplied as the isothionate, methanesulfonate (Lomidine®) or hydrochloride salt. Prior to the increased clinical use of PMD in North America for the treatment of AIDS associated *P. carinii* pneumonia,[294] little was known about this compound. As with most amidines and diamidines, PMD and its analogues have a broad spectrum of activity, inhibiting a plethora of processes within tumor cells,[295-299] MRSA,[300] *A. Keratitis*,[264] trypanosomes,[255,301-303] plasmodia,[304,305] leishmania,[304,306-310] *Crithidia fasiculata*,[311] *S. aureus*,[311,312] *C. neoformans*,[313] C. *albicans*,[314-317] *Escherichia coli* K12,[318] *Leptomonas sp*,[319] *Fusarium*, [320] *Babesia microti*,[321] *Aspergillus*,[322] *T. vaginalis*,[323] *Scedosporium prolificans*[324] and *Pneumocystis carinii* pneumonia.[297,325-328] In addition, PMD and PMD analogues have been shown to be NMDA receptor antagonists, neuroprotective *in vitro* from NMDA toxicity.[329,330] An ability to interact with DNA is widely reported in the literature for diamidines[245,284,331-337] with PMD in particular found to be 60 times more potent than cisplatin, inducing a higher frequency of programmed cell deaths associated to the inhibition of DNA synthesis by binding to DNA causing conformational changes.[338] Furthermore, PMD was found to interact with the regulatory protein ubiquitin causing alterations to its conformation and a 6 % increase in β-sheet content.[338]

2.5.2.1 Trypanosomiasis

Since the work of Ashley, PMD has been used for the treatment and prophylaxis of human African trypanosomiasis effective *versus T. brucei. gambiense* infection only.[255,301] PMD is rapidly absorbed by trypanosomes and is lethal both *in vitro* and *in vivo* being specific for the parasite rather than the host cell.[311] It has been shown that prolonged exposure to PMD *in vivo* may be of importance since the exposure time affects the sensitivity of the parasite.[339] PMD is parasiticidal against trypanosomes, effective in the early stages of the disease prior to CNS infection due to an inability to permeate the blood brain barrier effectively.[301,302] Although studies in patients with *T. brucei. gambiense* excluding involvement of the CNS have shown that after the last dose the cerebral spinal fluid contains small amounts of PMD.[302] Therefore PMD could be used in the early-late stages of the disease, a hypothesis that has been confirmed clinically.[340]

2.5.2.2 *Pneumocystis carinii* pneumonia

Pneumocystis carinii pneumonia (PCP) is a disease mostly observed within the immunosupressed host, particularly those suffering from Acquired Immune Deficiency Syndrome (AIDS) were PCP is one of the most common AIDS-defining diagnoses.[325] In 1958, the efficacy of PMD *versus* PCP was revealed.[341] Since then, PMD has found routine use in the treatment of PCP.[325] Furthermore, many diamidine analogues have been developed and screened as anti-PCP agents in the hope that they may circumvent the adverse reactions associated with the clinical use of PMD in PCP treatment that can cause severe morbidity and sometimes mortality.[342] However, little development had been made within the area of PCP treatment with more than 50% of patients under PMD treatment suffering from adverse effects[343] until the recent development of DB75 **49** and the orally bioavailable prodrug DB289 **50**.



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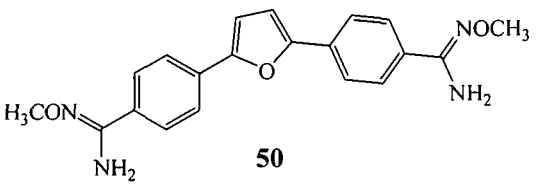

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To date, human trials suggest that DB289 is well tolerated and clinically efficacious against PCP and Pneumocystis jiroveci pneumonia.[344]

2.5.2.3 Malaria

PMD has never been used for the treatment of malaria although its activity is reported[345,346] therefore the antiplasmodial properties of PMD will be reviewed later within section 2.6.

2.5.2.4 PMD Toxicity

Due to poor oral bioavailability,[347] PMD was administered through slow intravenous (i.v) injection, however when administered this way, hypotensive reactions and subsequent collapse due in part to the liberation of histamine have been observed, thus i.v administration is no longer recommended.[348] To avoid the possibility of immediate toxic reactions associated with iv administration, the drug is now given intramuscularly to humans.[349] PMD administered intravenously is reported to be painful, can cause sterile abscesses, sciatic nerve damage or gangrene.[301]

Other adverse effects reported with PMD use include fainting, hypotension,[350] rash[351] and cardiac toxicity.[352] In addition, nephrotoxicity to PMD is reported,[353-356] understood to be due to the ability of PMD to accumulate and localize significantly in the kidneys[357] furthermore, PMD-induced acute pancreatitis is also observed clinically.[342] That being said, insulin dependent diabetes is most commonly reported within the literature following PMD use.[358-360] PMD-induced hypoglycaemia associated with high plasma insulin concentrations due to inappropriate insulin release and B cell destruction are reported.[359,361] This process generates insulin dependent

diabetes mellitus,[362,363] the effects of which are irreversible, dose and time dependent.[363] In certain cases, an increased insulin requirement even when PMD treatment ceased was noted.[358]

Toxicity to PMD has been linked to the metabolic oxidative cleavage of the ether oxygens.[364] A means of circumventing PMD associated toxicity seems to be within the route of administration. Aerosolised PMD is now recommended for PCP treatment with studies confirming enhanced tolerance in HIV infected patients.[365] Although this route does not appear to be applicable to trypanosomiasis and leishmaniasis presumably due to an inability of this route to target blood stage protozoa whereas PCP is localised within the lungs.

2.5.3 PMD Uptake in Malaria Parasites

2.5.3.1 Introduction

PMD and other diamidines accumulate significantly within the infected erythrocyte[242,366] which begs the question, how does this dicationic drug molecule traverse the cell membrane? Specific pathways are understood to mediate the accumulation of PMD within leishmaniasis, trypanosomiasis and the new permeability pathways within the malaria parasite.[242,367-371]

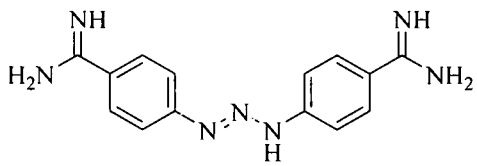
Pathways that mediate the uptake of nutrients into infected red cells are known processes we aim to target and therefore their properties will be discussed with respect to PMD and *plasmodium* induced permeability pathways. Out of interest, PMD uptake within trypanosome and leishmania infected cells will be briefly discussed.

2.5.3.2 Trypanosomiasis and Antimony Resistant Leishmaniasis

The specificity of diamidines for infected cells is important when considering the amidine moiety as our choice of cation. Although we are not using the trypanosomal system, it is important to be aware of their properties within this system also. It is known that cationic drugs can accumulate into cells and concentrate in the mitochondria to concentrations up to 10,000

fold.[366] This level of accumulation cannot be due to simple penetration through a pore. Many mechanisms have been proposed for this accumulation,[372] the most reasonable being that the differences induced within the cell membrane by the parasite play a role within the specificity of the drug for these cells, since it is known that diamidines are bound strongly by specific phospholipids.[373,374]

It has been shown that the PMD transport system in *T. brucei* and *Leishmania* parasites is carrier mediated[311,370] showing substrate specificity and high affinity for the amidine moiety of PMD.[311] Specifically, PMD and other diamidines have been shown to be transport substrates for the P2 aminopurine transporter.[368] In addition, the diamidines PMD and berenil **51** have a high affinity for the P2 transporter with little affinity for the P1 or H2 purine transporters[375] even though it has been shown that P2 transports only adenine and adenosine whereas P1 has a broader specificity.[376,377] It has been shown that in addition to the P2 transporter, PMD uptake is mediated by other transporters termed the high affinity PMD transporter (HAPT1) and the low affinity PMD transporter (LAPT1) in addition to the adenosine-sensitive PMD transporter (ASPT1).[371,378]



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2.5.3.3 Malaria; New Permeation Pathways

The transporters of the malarial parasite and parasitised cells are of potential use as drug targets by inhibiting the uptake of essential molecules or as routes for the delivery of drug molecules, giving selectivity to parasitised rather than host cells. Upon parasitisation of the erythrocyte, various changes are induced within the cell, the most notable of which are new permeation pathways (NPP) occurring 10-20 hours post invasion.[379] In order to proliferate and develop, the intraerythrocytic period of the parasite life cycle produces 16-32 offspring asexually over 48

hrs.[380] To facilitate this, the parasite by its very nature is dependent on the acquisition of the necessary essential materials from the host cell and host environment, since the parasite is devoid of the metabolic equipment required for their synthesis.

Evidence for the presence of NPPs comes from the fact that in order to maintain parasite growth, haemoglobin is digested to obtain amino acids,[381] choline used for phospholipid synthesis,[143] and purine bases for the synthesis of nucleic acids[382] using glucose for energy.[383,384] Aside from haemoglobin which is abundant within the cell, these materials must be acquired from their host environment into the erythrocyte and indeed the parasite itself. Interestingly, other nutrients have also been identified,[112,385] particularly for use in culture.[386,387] Furthermore, the infected erythrocyte has been noted to have a high rate of lactate production,[388,389] particularly at the schizont stage of reproduction.[388] This large rate of lactate production observed by the acidic pH within the food vacuole (pH 5.5)[390] is important since additional pathways must be in place to prevent acidification of the parasite and host cell.

These processes impart a necessity for the parasite to acquire these essential molecules from its host whilst securing the removal of waste from the cell. This is achieved by modification of the red cell membrane enabling the increased transport of solutes in and out of both the parasite and host cell. The result of which is a modified erythrocyte cell membrane with an increased permeability to a diverse range of low molecular weight solutes,[379,391] phosphorylation of which is believed to trap these compounds within the cell.[112]

Knowledge of this increased permeability to solutes increased speculation within the scientific community that these processes could be targeted as a means of enhancing drug delivery into infected host cells while avoiding drug accumulation into healthy cells. Thus knowledge of the features of the NPP is important to our choice of dicationic moiety within the drug template and therefore will be discussed.

2.5.3.4 Properties of the NPP

Malaria infected human erythrocytes have been shown to have a broad specificity[379] and increased permeability to organic cations[391] and low molecular weight solutes *via* transporters different to those of healthy erythrocytes and more similar to chloride channels (Cl^-) in other cell types.[392,393] Therefore, akin to Cl^- channels, there is an expectation for them to be permeable to cations, the magnitude of which would be a function of the counter-anion present. However, it has been noted that they are anion selective with significant cation permeability[394] resulting in an increased permeability to a range of inorganic and organic monovalent ions (both cations and anions), zwitterions, and nonelectrolytes.[379] The increased access of a variety of low molecular weight solutes *via* NPPs gives substantial permeability to a range of monovalent organic (quaternary ammonium) cations, the largest having an estimated minimum cross-sectional diameter of 11–12 Å.[391] Cations both small[379] and large[379,391] have been shown to pass through the NPP.

Staines *et al.* found that the rate of cation permeation was dependent on the nature of the anion present with no clear relationship between the permeation rate and size or hydrophobicity of these solutes showing a preference for small hydrophobic ions over larger or less hydrophobic solutes.[391] Indications that the NPP is a channel come from the fact that it has broad specificity absent of discrimination of isomers[393] and it does not saturate with increasing substrate concentration.[392] Other attributes of the NPP include; flux blockade by a variety of reagents, it is nonsaturable, the activation energy for the transport of solutes is lower than that typical of carrier mediated transport but typical of that for a diffusive process, there is no discrimination between the enantiomeric forms of permeating solutes (though they do show differential sensitivity to the *R*- and *S*-enantiomers of several optically active arylaminobenzoates), they have a general preference for anions and electro-neutral solutes over cations, are able to discriminate between alkali metal cations, and the rate of cation permeation is dependent on the nature of the anion present in the suspending medium.[379]

PMD has been shown to accumulate into malaria infected red cells only.[367] Interestingly, the accumulation of PMD into parasitised red cells can be blocked by inhibitors of haemoglobin

digestion[367] suggesting that PMD is a hematin binder. Indeed, a range of diamidine compounds have been shown to bind to FPIX and inhibit hemozoin formation *in vitro*. PMD uptake has been shown to be selective, sharing characteristics with the NPP due to two transport factors; an initial rapid non-saturable phase is observed for PMD uptake and the nature of the counterion has an effect on PMD transport ($Cl^- < Br^- < NO_2^- < SCN^-$).[367] Furthermore, *bis*-quaternary ammoniums have also been shown to bind to FPIX in the digestive vacuole being critical for both the accumulation and activity of these drugs.[157]

Choline is an essential nutrient required by infected cells, the transport of which is increased upon parasitisation of the red cell. Choline entry occurs by a facilitated-diffusion system involving an endogenous carrier, or through the NPP, this high increase in choline transport activity related to modifications in choline carriers and/or in their environment after *plasmodium* infection.[379,395] The mechanism by which choline penetrates the intracellular parasite is not fully understood however, it has been shown that a parasite choline transporter is responsible for the uptake of choline into the intracellular parasite and not the host cell choline carrier. Furthermore, antimalarial choline analogues are transported by the parasite choline carrier and not the host cell carrier. In addition, *bis*-amidines and *bis*-quaternary ammoniums are acquired by a cooperative transport system between the induced permeability pathways in the host erythrocyte membrane and the parasite choline transporter as shown in Figure 9.[164] A factor that is very important within structured drug design since presumably in addition to the IPP, there is the possibility of additional routes of drug uptake by the parasite. However, it is not clear through which route choline inhibition by amidines occurs,[156] through the endogenous carrier, the parasite choline carrier or the NPP.[164]

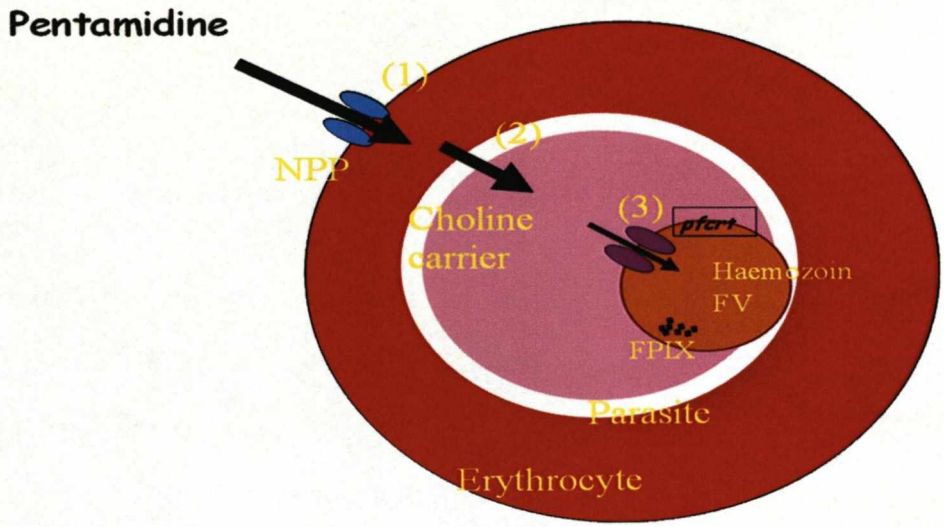


Figure 9. Schematic representation shows the transport of drug and binding to FPIX. Definitions: (1) NPP; New Permeation Pathway, (2) Choline Carrier, (3) *pf*crt; *Plasmodium falciparum* chloroquine resistant transporter.

It should however be noted that the antimalarial activity of amidines may not be related solely to uptake since if activity is dependent on uptake through the NPP, this does not explain why amidines shielded by aliphatic groups are less active than unsubstituted amidines[304,396] (as shown in Figure 10) due to the preference of the NPP for compounds with an increased hydrophobicity.[391]

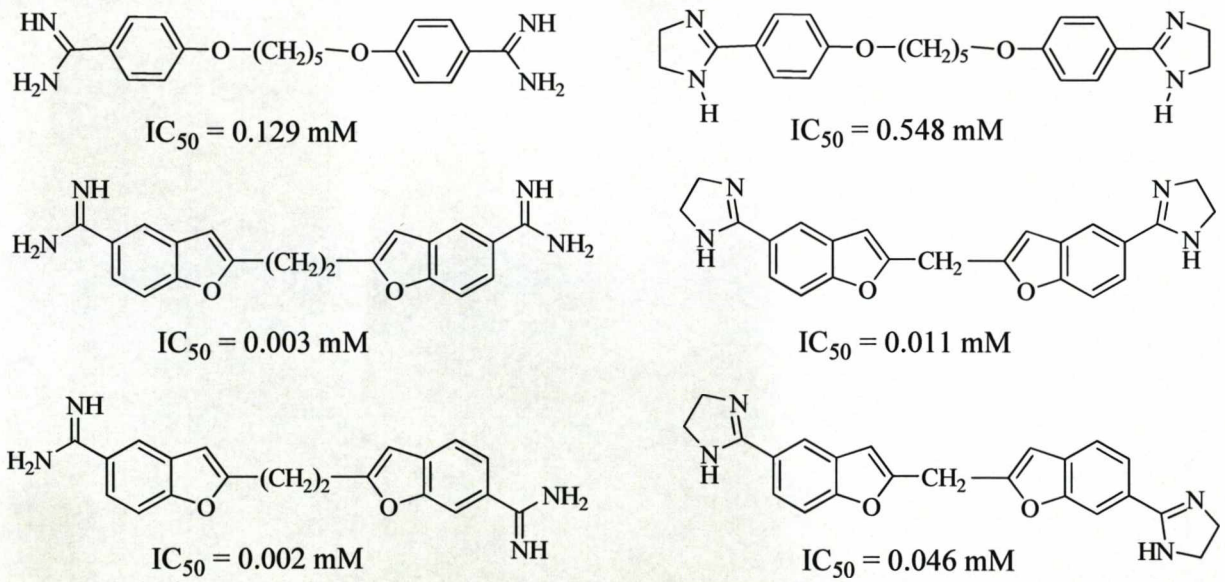


Figure 10. Activity of shielded diimidazolines *versus* diamidines against CQ resistant parasites

2.5.4 PMD- Pharmacokinetics and Metabolism

Pentamidine pharmacokinetics exhibit inter-individual differences in maximum plasma concentrations as well as pronounced drug accumulation[302] with a long elimination half-life ≥ 4 days,[397] an issue when considering the toxicity issues associated with PMD use. The metabolism of PMD (Figure 11) is important for the rational design of compounds in the hope of circumventing PMD associated toxicity. It has been shown that PMD is rapidly metabolised *in vivo* by P450 dependent mixed-function oxidase mediated mechanisms[398-400] to at least seven primary metabolites with *N*-hydroxylation resulting in a loss of activity.[398,401] Therefore with respect to activity, it appears metabolism is deactivating.

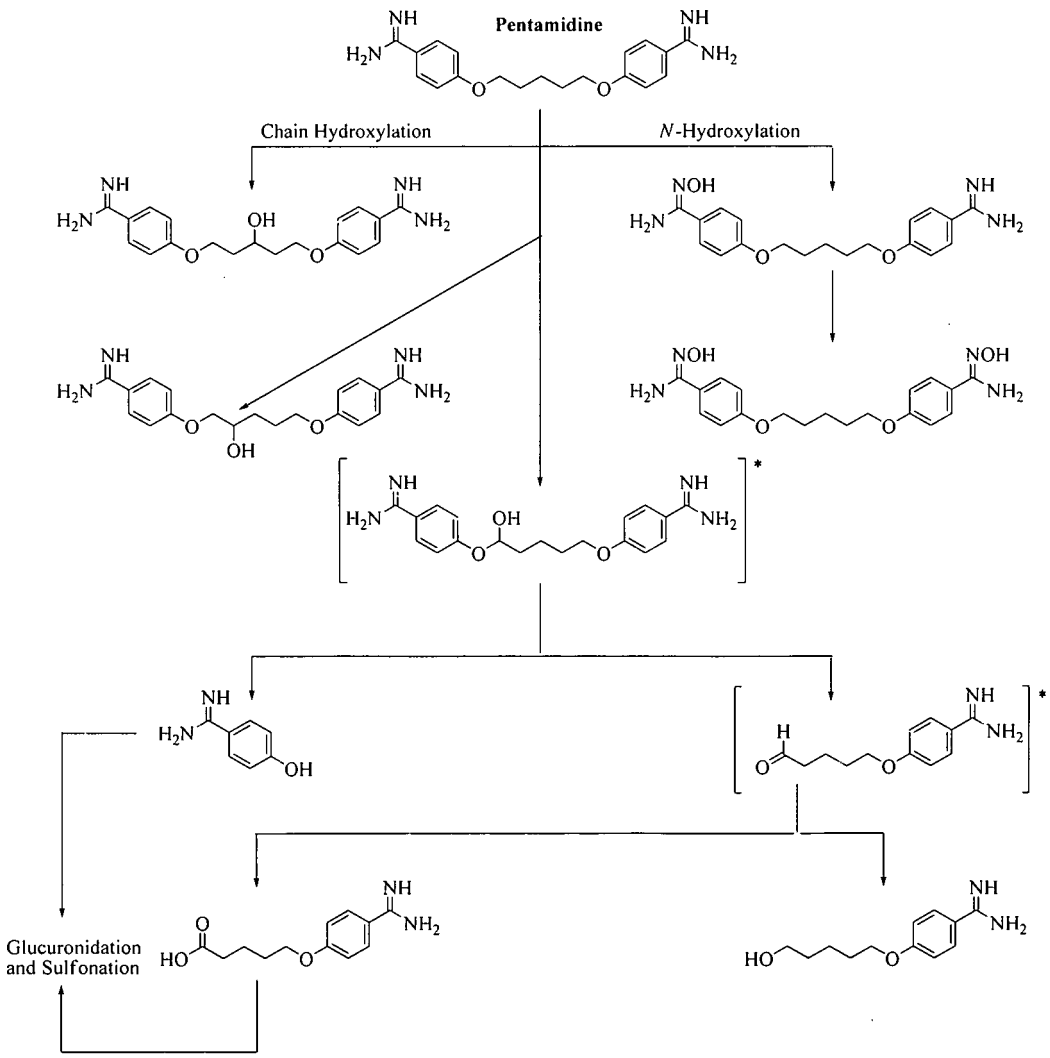


Figure 11. Metabolism of PMD (brackets and asterisks indicate compounds that have not been isolated)[343]

2.5.5 Resistance

Searching the literature, it is clear that little work has been done regarding resistance mechanisms for PMD or diamidines for that matter, and malaria. There are however many citations regarding resistance in the trypanosome which will be covered briefly.

Since trypanosomes are unable to metabolise PMD,[400] metabolism based mutations can be ruled out as a resistance mechanism in the parasite. Diamidine-arsenical cross-resistance has been observed experimentally and appears to arise from alterations in the P2 transporter[402-404] since the P2 transporter has been shown to accumulate melaminophenylarsenicals also.[377] Additional evidence for the shared transport system between melaminophenyl arsenicals and diamidines comes from the varying degrees of cross-resistance to diamidines in the order stilbamidine > berenil > propamidine > PMD[405] further suggesting that these compounds are recognised by the same transport system. Trypanosomal resistance to diamidines may therefore be due to alterations in the P2 transporter causing decreased accumulation of drug[368] although it is noted that resistance to arsenicals and PMD is not as pronounced as to diminazene (berenil) indicating the presence of other transporters for the residual uptake of melaminophenyl arsenicals.[369] The presence of more than one transporter could explain why some parasites without P2 activity have been shown to be sensitive to PMD.[406]

It is probable that high-level resistance is due to loss of more than one transporter and in the case of mutations at one or more of the transporters, the uptake of PMD may be unaffected since there is the possibility of switching over to one of the other transporters. It is therefore likely that transport/uptake mediated resistance for PMD will take longer to develop than for those drugs whose uptake has been shown to involve one transporter such as the melaminophenyl arsenicals. Despite its widespread use as a prophylactic, resistance to PMD has not been a significant problem in the field.[242] This is illustrated by the fact that *T. brucei rhodesiense* strains from west and central Africa isolated in the periods 1960-1996 and 1999-2004 showed little difference in IC_{50} values to PMD.[407]

2.5.6 Mechanism of Action

The mechanism of action of diamidines is unclear and heavily debated since they are active against many targets, therefore the elucidation of their major mode of action is difficult. It is however generally accepted that more than one mechanism may be taking place. PMD itself has been shown to interact with systems such as the respiratory chain within mitochondria,[310] uncoupling of oxidative phosphorylation in rat liver mitochondrion,[366] nucleolar dispersion, aggregation and loss of ribosomes,[374] *in vivo* and *in vitro* inhibition of dihydrofolate reductase,[349] irreversible inhibition of monoamine oxidase enzymes *in vivo*,[408] disruption of amino acid accumulation and oxygen consumption,[311] inhibition of DNA, RNA, and protein synthesis,[295,311,312] and the inhibition of group I intron splicing.[315,316] In addition, diamidines have been shown to disrupt mitochondrial function[409] inducing damage to the mitochondrial membrane with lesions apparent 5-6hrs after treatment,[374] loss of cytoplasmic ribosomes[373,374,410] disintegration of the kinetoplast DNA core[374] and the inhibition of cytosolic oligopeptidase action.[411]

The biochemical changes induced as a consequence of these effects with respect to cell death do however remain indefinable as are the interactions between diamidines and DNA. It is the interactions of diamidines with DNA that are widely reported within the literature.[245,284,335,412,413] The role that binding to DNA plays within the antimalarial activity of diamidines is unclear and largely un-linked by reports within the literature since mostly, DNA binding affinity is calculated with respect to antitrypanosomal activity. Though few papers report DNA binding affinity alongside both antimalarial and antitrypanosomal activity,[414,415] the link between DNA binding and antimalarial activity remains unresolved.

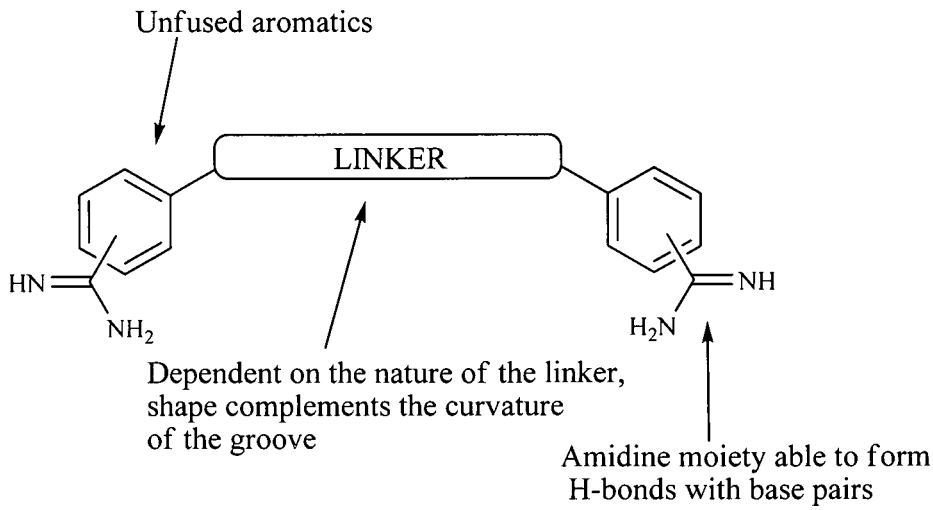


Figure 12. DNA-binding properties

Studying the aryl diamidine structure shown in Figure 12, it is clear that the structure contains properties of a typical DNA binder, the binding mode is however a matter of debate. It is generally accepted that the binding of *bis*-amidines to DNA occurs at the AT-rich (adenine and thymine base pairs) region of the minor groove, an area where the interactions are governed by shape, distribution and dimension properties manifested in the isohelicity to DNA through the nature of the linker between the two amidine moieties, amidine H-bonding and the lipophilicity of the alkyl linker.[416] It is largely accepted that the binding of aryl diamidines to DNA is of a non-intercalatory manner at AT-rich sites with intercalation at GC-sites,[417] the major interaction being at the AT-rich region of the DNA minor groove.

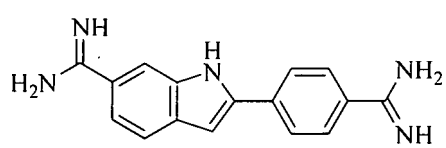
With respect to PMD, this interaction is understood to be intercalatory at the AATT motif in a noncovalent manner[418] through the formation of H-bonding and Van de Waals interactions between the amidines and base edges.[336] However, extensive studies have also shown that PMD has the ability to bind at the minor groove of DNA with a strong preference for AT-rich sequences, in a non-intercalative manner.[419] Van Dross and Sanders observed that PMD does not target topoisomerase I or inhibit the catalytic activity of topoisomerase I nor does it stimulate topoisomerase I mediated DNA cleavage.[420] PMD does however promote the linearization of minicircle DNA at therapeutic levels.[421] Furthermore it has been demonstrated that PMD binds to

tRNA through non-specific hydrophobic interactions inhibiting aminoacylation and translation, challenged by replacing the oxygen moiety with sulphur which is less able to hydrogen bond.[422]

The ability of PMD and its analogues to recognise and selectively bind to AT-rich sequences within DNA is dependent on the length of the alkyl linker. Studies with analogues of PMD found that butamidine, hexamidine, octamidine and nonamidine were poorly sequence selective[334] whereas propamidine, PMD and hexamidine have been found to bind selectively at AT-sites with propamidine binding more strongly than PMD which binds more strongly than hexamidine.[423] Understandably, geometry is also a function of binding with *cis* isomers binding more strongly than their *trans* counterparts.[333,424]

Cationic diamidines are known to enter the kinetoplast within minutes of treatment,[425,426] possibly due to its increased content of A and T compared to nuclear DNA.[374] The binding interaction involves the formation of a 'close-fit' with the convex floor of the minor groove through bending of the molecule to optimise contacts between drug and AT base pairs.[423,427]

DAPI **52** is known to inhibit protein synthesis,[428] the relaxation of DNA[429] and interact preferentially with A and T bases of double stranded nucleic acids, though this interaction is non-intercalative and reversible.[418] Two modes of binding are evident, at the A and T bases and also external stacking absent of base specificity of an electrostatic nature.[430] Although the precise mode is not fully understood it is believed to consist of a strong, specific H-bonding interaction between the amidino and base pairs. DAPI should therefore be viewed as an intercalator that has unusual and very favourable interactions in the minor groove at AT sequences.[431]



52

DB75 and other furans appear to bind to DNA by two modes; strong minor groove binding interactions at AT sites[432] due to favourable AT specific DNA binding and intercalation at GC

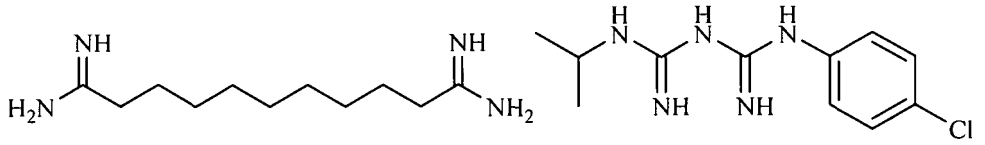
containing sequences.[433] Furthermore, DB75 makes direct hydrogen bond interactions with the $d(CGCGAATTCGCG)_2$ DNA sequence through both amidinium groups to oxygen atoms of thymine bases.[434]

The role of DNA binding on the absolute mechanism of parasite death is unclear nevertheless, it is largely accepted that DNA binding results in the cessation of a number of DNA dependent enzymes accompanied with inhibition of transcription processes.[224] The specificity of the cationic amidine moiety for A and T sequences is presumably due to their stronger negative zones of electrostatic potential.[435] There is also a belief that these compounds are able to bind in a way that causes the DNA-drug complex to be unstable and eventually destroyed. Therefore the additional cellular effects noted earlier, are suggested to be secondary to DNA binding.

The role of DNA binding within the antimalarial mode of action remains unclear. Since the elucidation of the genome and discovery of AT-rich super-islands, there is a cause to explore these properties in more detail within the malarial parasite, since it is clear that these compounds have an affinity to AT-rich biological sites.[15]

2.6 Diamidines as Potential Novel Dicationic Antimalarial Chemotherapeutics

The possibility of transferring malaria parasites to canaries, achieved in 1926,[3] led to the assessment of antitrypanosomal drugs *versus* malaria. In 1938, Christophers assessed the activity of undecane diamidine **53** showing that a daily dose of the diamidine could treat an infection of *P. knowlesi*.[436] The development of the guanidine containing compound paludrine (proguanil hydrochloride) **54**, in 1944, a result of war-time research led to a brief investigation into diamidines and diguanidines as antimalarials since paludrine displayed excellent antimalarial activity, having the advantage that it was less toxic than other compounds and could be used for both treatment and prophylaxis.[3]



53 **54**

However, the low cost and efficaciousness of CQ resulted in a preference and reliance on its use by clinicians and patients, thus diamidines such as **53** were not investigated for antiplasmodial use. However, the recent emergence of widespread resistance to CQ (among other antimalarials) has caused treatment failures leading to a requirement for novel agents able to circumvent resistance pathways due to their originality.

PMD was naturally the starting point of investigations into diamidines as antimalarials since PMD is widely used for the treatment of protozoal infections with a long history in this area. Furthermore, it has long been known that patients treated for kala azar with PMD or stilbamidine rarely suffer from malaria even when in endemic areas.[242] Consequently, experiments have been performed using both CQ resistant and susceptible plasmodia finding that PMD is concentrated 500 fold by erythrocytes infected with *P.Falciparum*.[367]

The antimalarial mechanism of action of PMD and other diamidines is not fully understood. Conceivable mechanisms include the inhibition of hemozoin formation. Mayence *et al.* used infrared spectroscopy and colorimetry to examine the interaction between ferriprotoporphyrin (IX) (FPIX) and diamidines in cell-free systems finding that *bis*-benzamidine-heme complexes are formed.[437] They found that amidines form adducts with FPIX, and that removal of the amidine function resulted in analogues that do not inhibit hemozoin formation. Furthermore, Stead and Bray studied the interaction between PMD and propamidine with heme, finding that PMD accumulation can be blocked by inhibitors of haemoglobin digestion, suggesting that PMD binds to FPIX. Both compounds were found to bind to FPIX, inhibiting the formation of hemozoin.[367] Of particular importance however, is the finding that binding to FPIX may be the main mechanism by which diamidines exert their antiplasmodial effect since inhibitors of haemoglobin digestion diminish their activity.[367] Furthermore the Bray group have shown that

diamidines inhibit hemozoin formation *in vitro* with a concentration-dependent potency similar to CQ as shown in Figure 13 (unpublished data).

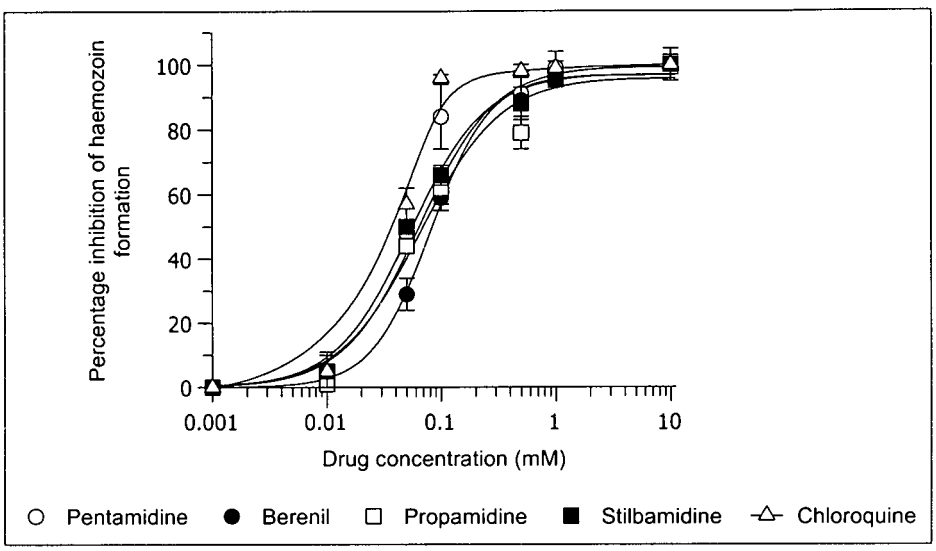
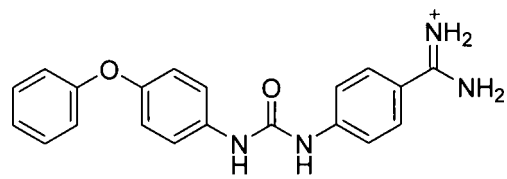


Figure 13. Inhibition of hemozoin crystal formation by diamidines.

When discussing the antimalarial mode of action for diamidines, hematin binding is a common talking point though there are suggestions that the *bc1* complex could also be a potential target for diamidine antimalarials.[328] The basis for this argument is plausible when combining the evidence; the mitochondrion is commonly linked to the biological effects of diamidines thus it is reasonable to suggest that they are effective in the malarial parasite and more pertinently, the *bc1* complex contains heme. Interactions between antimalarials and the *bc1* complex are known[438-440] it seems however that study within this area concerning diamidines is slight therefore these claims as yet cannot be substantiated.

2.6.1 Amidine Containing Molecules with Antiplasmodial Activity

Although this compound is *mono*-cationic at physiological pH, the diphenylurea amidine derivative WR268961 **55** has been included out of interest.



55

Malarial parasites use the aspartic proteases, plasmepsins I and II to digest haemoglobin.[85,441] From a range of compounds screened from the Walter-Reed database, WR268961 was found to inhibit *P. falciparum* growth *in vitro* at concentrations between 0.03 and 0.16 μg/ml. Interestingly, WR268961 is selective for *P. falciparum* plasmepsin II and *P. vivax* plasmepsin while being a poor inhibitor of human aspartic protease, thus making the molecule selective for malaria parasites rather than mammalian cells with parasites unable to accumulate intact haemoglobin upon treatment with WR268961.

Similar compounds absent of the amidine moiety were also assessed. Although they showed specificity for plasmepsins rather than human proteases, they were poor inhibitors of plasmodial growth (IC_{50} typically 100 times greater) and lacked specificity over host cells. These results suggest that the amidine moiety may play a part in the binding of the compounds to plasmepsins however, docking experiments show that it is the urea core that interacts with the plasmepsin active site as shown in Figure 14.[442]

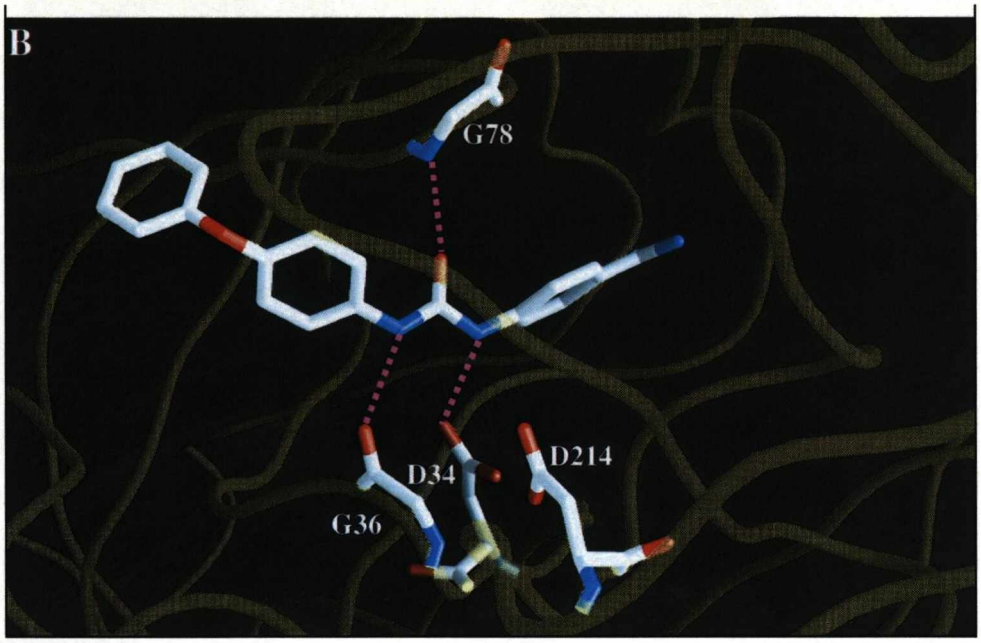
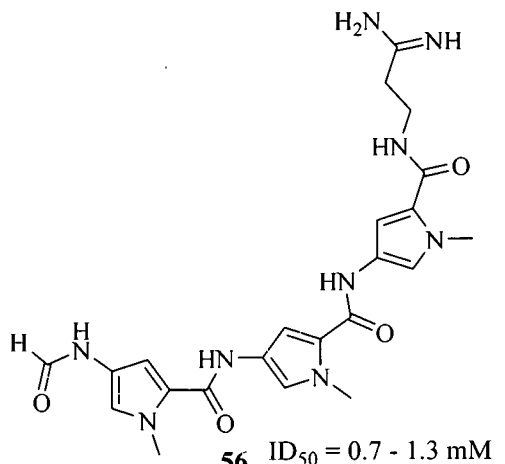


Figure 14. Model of WR268961 bound to plasmepsin. The main chain of the plasmepsin enzyme is represented by a semitransparent yellow ribbon. The side chains of four plasmepsin residues involved in hydrogen bonds (purple dashed lines) are coloured by atom type.[442] Red = Oxygen, White = carbon, Blue = Nitrogen.

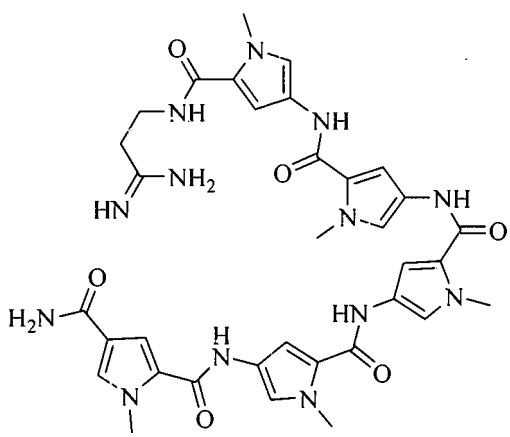
This suggests that additional mechanisms are in play since the basic amidine containing compound WR268961 is the only diphenylurea derivative of the series that not only prevents parasite growth but also plasmepsin activity. Furthermore, Bhattacharaya and co-workers have synthesised amidine-containing derivatives of WR268961 against erythrocyte-stage *P. falciparum* again finding that amidine containing diphenylureas gave the most active compounds.[443] Presumably hematin binding is the additional mode operating here, although studies regarding the ability of these compounds to inhibit hemozoin formation have not been performed.

The tripyrrole amidine distamycin A **56** is a natural antibiotic isolated from *Streptomyces distallicus* cultures[444] that possesses antibacterial and antiviral properties.[445] It is a known minor groove binder (non-intercalative) with high sequence specificity to AT-rich sequences in DNA[446,447] *via* electrostatic interactions, hydrogen bonds (drug-DNA) and Van der Waals contacts as drug-drug and drug-DNA stacking interactions with base pairs, disrupting replication and transcription.[448] It is not cytotoxic against tumour cells and thus has been used as a carrier for targeting cytotoxic alkylating moieties in the minor groove of DNA.[449]



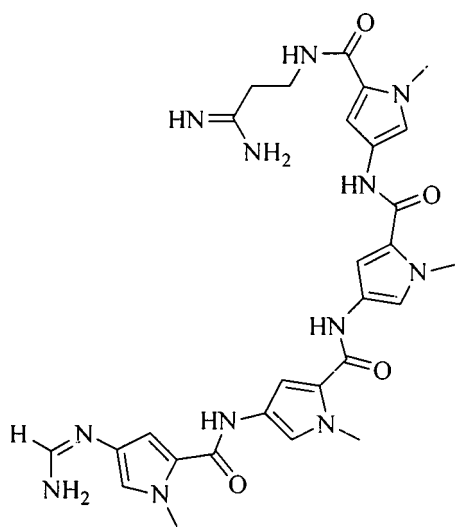
56 ID_{50} = 0.7 - 1.3 mM *versus P.f alciparum*

Distamycin has shown antimalarial activity *in vitro* against CQ sensitive and resistant strains of *P. falciparum* however there are toxicity issues with its use. Lombardi *et al.* synthesised analogues of distamycin, the most active compound being compound **57** giving 100 % inhibition of parasites after 4 hrs incubation *in vitro*. Cytotoxic testing showing the analogue to have a low toxicity shown in Figure 15 as the dose required to reduce a given biological effect by 50% (ID_{50}).[260]



57

ID_{50} = 0.25 μM

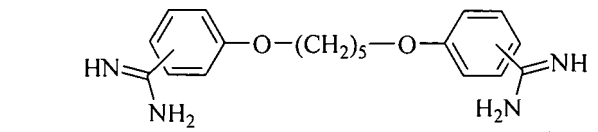


58

ID_{50} = 0.3 μM

Figure 15.

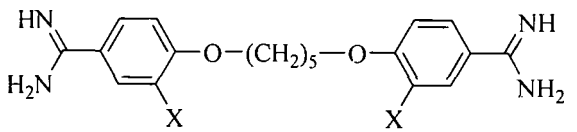
Analogues of PMD were synthesised by Bell and co-workers exhibiting antimalarial activity *versus P.falciparum*.[306] The position of the amidine functionality clearly plays a role within the activity of these compounds with the *p*-amidine functionality (PMD) giving the most potent compound *versus* CQ sensitive parasites though the *m*-amidine appears to avoid any *pfcrt* based efflux resistance mechanisms as demonstrated by the IC_{50}s shown in Figure 16.



***p*-Am**		***m*-Am**
IC_{50} = 0.129 μM	CQ resistant	IC_{50} = 0.085 μM
0.051 μM	CQ sensitive	0.082 μM

Figure 16. Effects of ring substitution

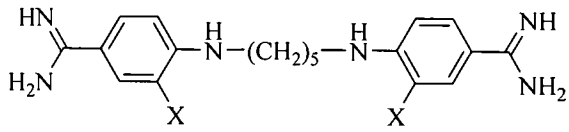
As shown in Figure 17, heteroatom substitution was also found to exert an effect on the activity of these compounds though not to a large extent with chlorine substitution producing the most marked effect on CQ sensitive parasites.



X	IC_{50} CQ resistant	IC_{50} CQ sensitive
NO_2	0.114 μM	0.124 μM
OCH_3	0.101 μM	0.108 μM
Cl	0.129 μM	0.080 μM
Br	0.146 μM	0.181 μM

Figure 17. Effects of heteroatom substitution

The effect of exchange of the oxygen moiety for nitrogen is shown in Figure 18 demonstrating an enhancement in activity for both resistant and sensitive parasites. Clearly, the nature of ring substitution has an effect on the activity of these molecules with the larger NO_2 groups causing a marked decrease in activity.



X	IC_{50} CQ resistant	IC_{50} CQ sensitive
H	0.045 μM	0.030 μM
NO_2	0.248 μM	0.109 μM
NH_2	0.183 μM	0.084 μM

Figure 18. PMD structure activity relationships

Figure 19 shows the effect the nature of the dication imparts on antiplasmodial activity. PMD gave an IC_{50} of 0.129 μM *versus* CQ resistant parasites and 0.051 μM *versus* the CQ sensitive strain however, the imidazole containing analogue causes a decrease in the potency of this compound. Furthermore, substitution on the aromatic ring for *O*-methyl groups results in an increase in activity for CQ resistant strains whilst giving a decrease in potency for CQ sensitive ones.

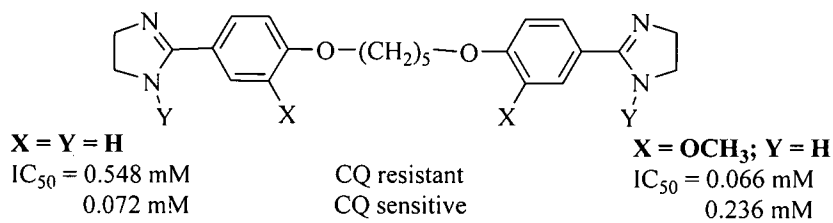
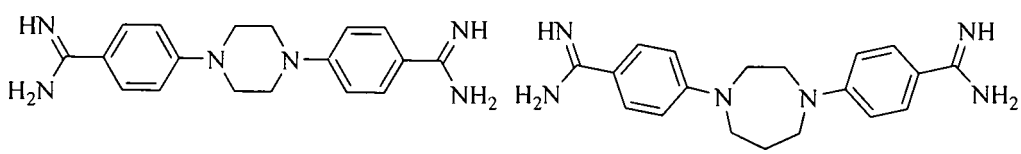


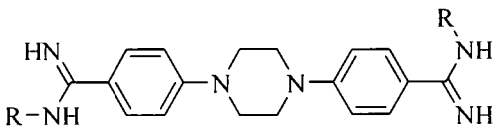
Figure 19. PMD structure activity relationships

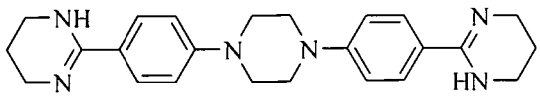
The flexible pentyldioxy linker in PMD was replaced with various restricted linkers by Huang *et al.* and tested for activity against CQ sensitive and -resistant *P. falciparum* strains *in vitro*. The most active compounds were *bis*-benzamidines linked with a 1,4-piperazinediyl **59** or 1, 4-homopiperazinediyl **60** moiety with IC_{50} values as low as 7 nM.[304]



59 **60**

Constraints have been imparted on the biphenyl PMD structure by introduction of a non-flexible core. Mayence *et al.* synthesised a range of *bis*-benzamidines and related congeners with activity *versus* CQ resistant and sensitive strains of *P. falciparum* the most active analogues are shown in Figure 20.





IC_{50} = 5 nM *versus* both strains yested

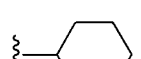
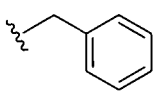
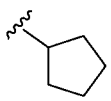
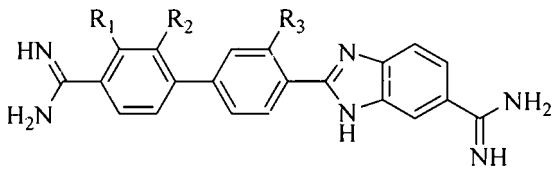
R	IC_{50} CQ sens	IC_{50} CQ rest	
H	3 nM	4 nM	Diamidine **61**
	3 nM	4 nM	
	3 nM	4 nM	
	4 nM	8 nM	
	6 nM	18 nM	
	3 nM	4 nM	

Figure 20. Conformationally restricted diamidines

The ability of these analogues to bind to DNA was also assessed. The parent compound diamidine **61**, has a strong binding affinity for DNA at AT-rich sequences, though when comparing the binding affinities of potent compounds with inactive ones, it is apparent that DNA binding does not correlate with activity, thus suggesting that DNA binding is unlikely to play a major role in the antimalarial activity of these compounds. Analogues were therefore assessed for an ability to act within the haemoglobin degradation stage of parasite maturity by interacting with FPIX finding that all benzamidines formed complexes with FPIX and inhibited the formation of hemozoin. However, analogues devoid of the amidine moiety did not inhibit

hemozoin formation in agreement with their IC_{50}s all of which were greater than 500 nM. Interestingly, some analogues had IC_{50}s within the same range *versus* both CQ resistant and sensitive parasites indicating that these compounds are unaffected by the *pfcrt* based efflux mechanisms deemed responsible for CQ resistance in *P. falciparum*. The fact that replacement of the amidine moieties leads to a loss in activity infers a requirement for basic, nonsterically hindered amidine moieties. The toxic potential of these analogues were also assessed finding that each of the active compounds had a low cytotoxicity.[305]

A series of near-linear biphenyl benzimidazole diamidines have shown potent antimalarial activity *versus P. falciparum*, exhibiting IC_{50} values ranging from 0.5 to 23 nM, the most potent of which are shown in Figure 21.[415] The mode of antimalarial activity is not discussed nor investigated however it is clear that these analogues display increased activity *versus P. falciparum* than their protozoan counterpart *T. brucei rhodesiense*, the most potent compound giving an IC_{50} of 3 nM in this genus.



	IC_{50} vs CQ rest K1
$R_1 = H; R_2 = H; R_3 = H$	0.5 nM
$R_1 = F; R_2 = H; R_3 = H$	1.0 nM
$R_1 = H; R_2 = H; R_3 = F$	1.0 nM

Figure 21. Dicationic near-linear biphenyl benzimidazole derivatives

As shown in Figure 22, several linear diamidines have been synthesised exhibiting potent activity *versus P. falciparum* the most potent giving an IC_{50} of 0.5nM.[337]

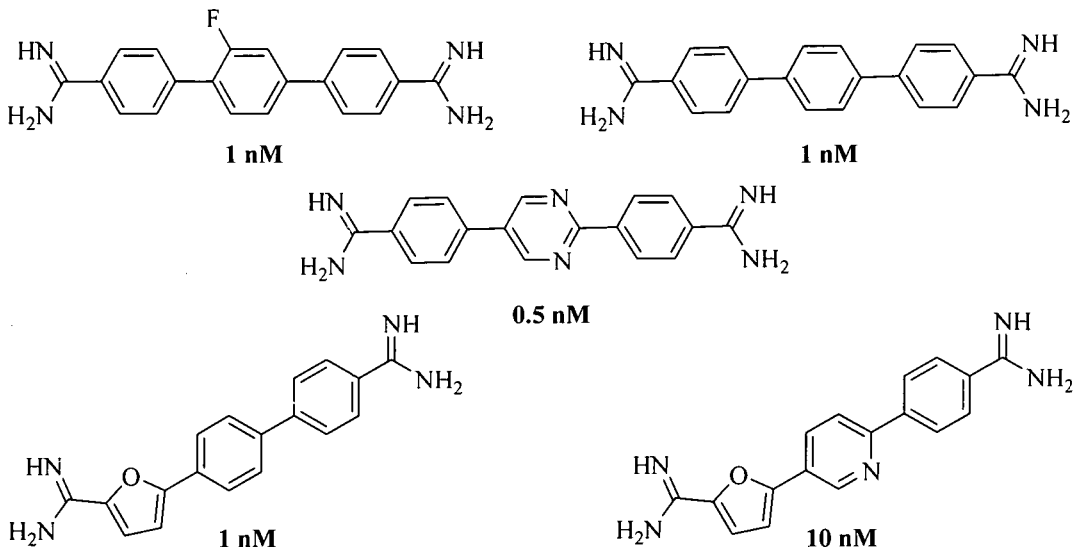
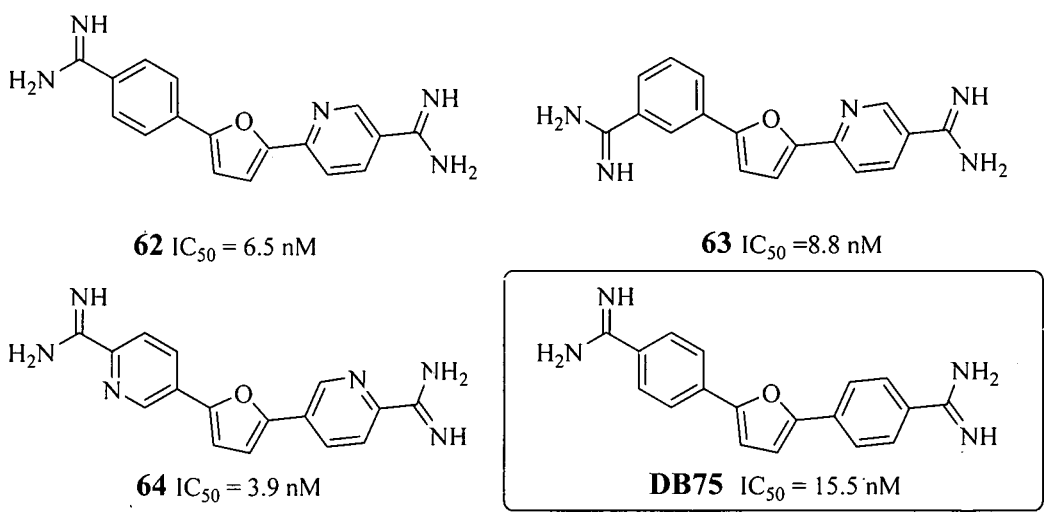


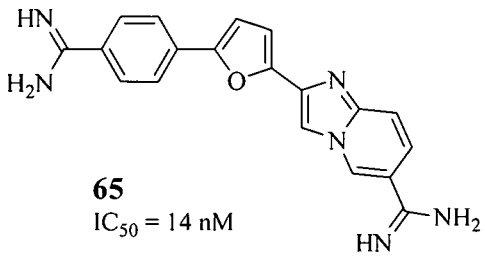
Figure 22. Antiplasmodial activity of the most potent terphenyl diamidine analogues *versus* K1 CQ resistant strain

Investigations into the mode of antiplasmodial activity were not undertaken, presumably fixing the structure results in an increased binding strength to the carboxylate residues of heme although the role of the nitrogen atoms within the central linker is unclear.

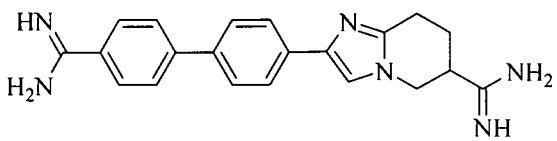
Diaza-analogues of the antiprotozoal agent DB75 have been synthesised with compounds **62**, **63** and **64** showing greater *in vitro* activity than the parent compound (DB75).[450]



Imidazo-phenyl based diamidines **65** and **66** have been synthesised showing activity *versus P. falciparum in vitro* the lead compounds inhibiting 50 % parasitic growth at a concentration of 14 nM.[451]



65
IC_{50} = 14 nM



66
IC_{50} = 14 nM

2.7 Summary

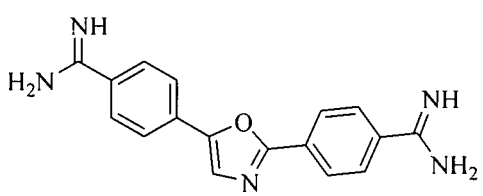
Essentially, the finding that diamidines can bind to DNA without associated antimalarial activity suggests that the mechanism of diamidine activity against *P. falciparum* may be different from those noted for trypanosomal and leishmanial parasite systems.

Clearly, diamidines possess antimalarial activity, some boast potent activity, and as such a portfolio of their antimalarial activity and properties is being generated. This has led to the emergence of diamidines as an antimalarial subclass in their own right. It seems evident, that the diamidine moiety would be a good choice of substituent for the development of antiplasmodial agents and therefore was the functionality chosen for our drug template. The DNA binding properties of these molecules are of concern in terms of their ability to exert this effect on healthy cells; however their specificity for infected cells over host cells leaves us positive that although this form of toxicity could indeed occur, it would be unlikely. Furthermore, the rate of DNA synthesis in parasitic cells is known to be much higher than mammalian cells.[142] It is probable therefore, that a compound whose mechanism of action involves targeting DNA would be much more damaging to the parasite than its mammalian host.

2.8 Other PMD Analogues with Antiprotozoal Activity

The ability for a compound to inhibit the processes of one protozoan form does not mean that it will be able to inhibit another form, observed clearly in the case of malaria and trypanosomiasis. However, out of interest other diamidines possessing antiprotozoal activity are reviewed from the perspective of evaluating active antiprotozoal diamidine structures.

Das synthesised cyclic amidines and diguanyls of furan, thiophene, and pyrrole giving activity *versus T. rhodesiense* in mice.[452,453] Within the same biological system, Das used oxazole and 1,3,4-thiadiazole heterocycles finding that the activity of the diamidines was greater than their cyclic guanyl analogues, the activity of compounds **67** and **68** being comparable to PMD.[454]



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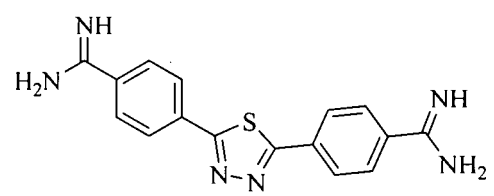
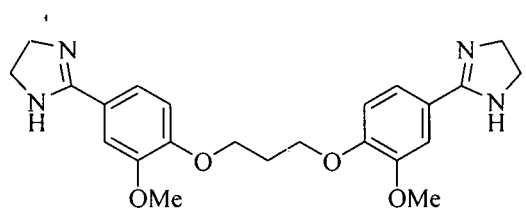
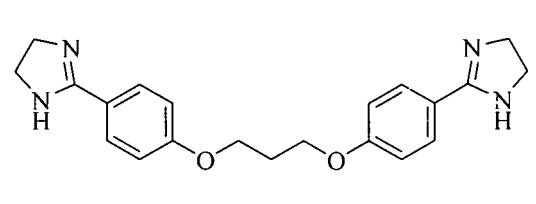


68

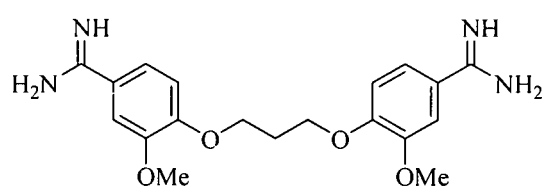
Jones exchanged amidine groups for imidazolines giving lead compounds DIMP **69** and DIPP **70** both of which are more effective and less toxic than PMD *versus* PCP. PCP infected rats treated with DIMP for 2wks at 1 mg/kg/day gave results equivalent to 10 mg/kg/day PMD.[341]



DIMP, 69



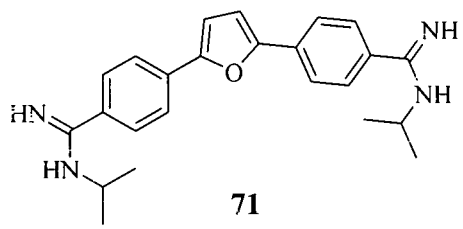
DIPP, 70



DAMP

DAMP and DIMP have also demonstrated activity *versus Giardia lamblia*[335] with DAMP being most active with an IC_{50} superior to two of the compounds used to treat giardiasis; metronazole and tinidazole.

Extended aromatics systems also exhibit anti-PCP activity. DB75 and extended derivatives **71** and **72** have been shown to exhibit activity against PCP with a lead compound approximately 100 times more effective than PMD itself. Substitution of an alkyl group onto the nitrogen of the amidino group gives a compound with an increased affinity for DNA attributed to the increased stability imparted on the DNA complex by the enhanced Van der Waals interactions. It is also interesting to note that a strong correlation between top II inhibition and anti-PCP activity is not observed for extended systems.[455-458]



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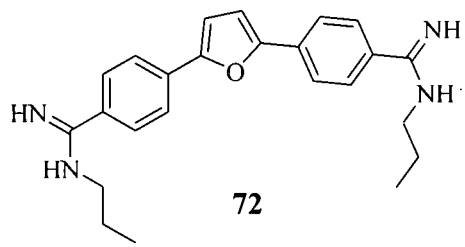
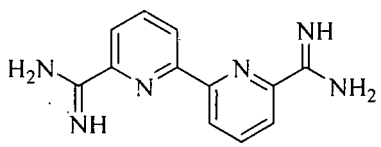
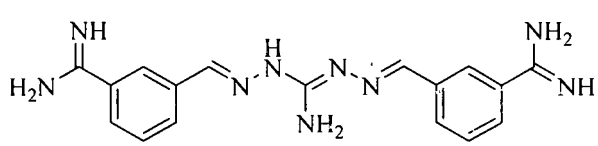


72

Diamidine analogues were assessed by Calonge *et al.* in order to assess their effect on polyamine metabolism and cell proliferation in *Crithidia fasciculate.* A structural requirement for two arylic groups rather than one was observed giving adducts **73** and **74** which were ten times more active against cell growth.[459]



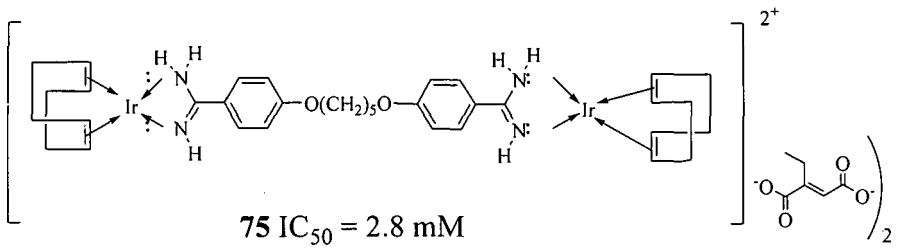
73 EC_{50} = 10.3 mM



74 EC_{50} = 8.7 mM

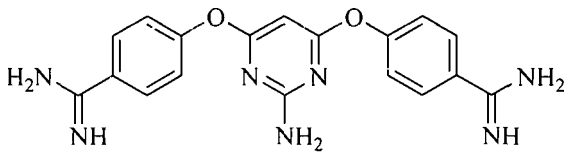
Organometallic compounds containing platinum have been shown to have trypanocidal activity.[187,460] Iridium and rhodium containing organometallic derivatives of PMD have been synthesised showing antileishmanial activity against *L. donovani* with some analogues giving

IC_{50}s better than PMD such as the Iridium (Ir) analogue **75**.[461] Furthermore, *cis*-Pt PMD iodide is active in a single dose both in mouse and sheep trypanosomiasis models.[462]



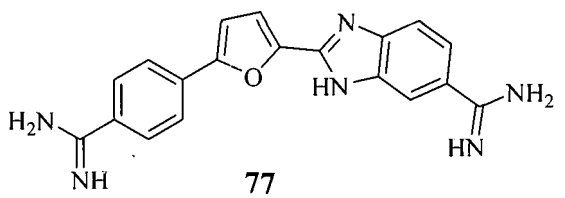
75 IC_{50} = 2.8 mM

Ring-based analogues of PMD with benzene, pyrimidine or pyridine at the centre have also been shown to be active against PCP in culture with benzene and pyridine generating analogues with anti-PCP activity, the pyrimidine analogue **76** being much less active.[463]



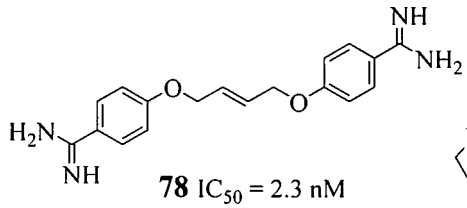
76

DB293 **77** is a benzimidazole analogue of DB75 which unlike previous analogues discussed, binds to both GC and AT base pairs in the binding site.[464,465]

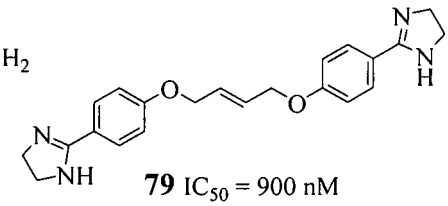


77

Donkor and colleagues synthesised a range of dicationic compounds related to PMD with trypanocidal activity. They found that diamidines are more potent than diimidazolines shown by diamidine **78** and diimidazoline **79** with antitrypanosomal activity decreasing when polar groups were introduced into the carba linker.[466]

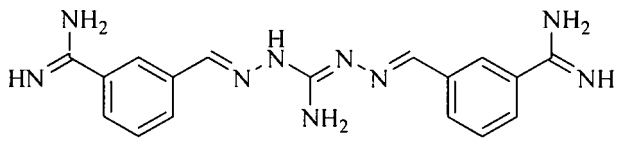


78 IC_{50} = 2.3 nM



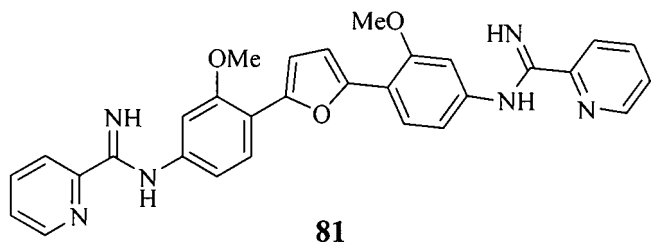
79 IC_{50} = 900 nM

When it comes to binding at the DNA minor groove it seems that sometimes there are no rules. The aromatic diamidine CGP 40215A **80** has a shape that does not match the curve of the groove, although it has been shown to bind strongly monomerically with a binding strength approximately six times higher than for berenil with the AATT hairpin.[467] These results are in accordance with the potent antitrypanosomal activity of CGP 40215A. This compound was active against multidrug-resistant trypanosomes with an IC_{50} of 0.0045 μM in addition, it was significantly less toxic than other compounds tested.[468]



80

A series of fixed long chain derivatives of PMD have been developed with activity against T. *cruzi in vitro*.[469] The lead compound DB709 **81** inhibits the intracellular stages of the parasite, a target that is hard to attack. In addition to the potency of this compound, toxicity to host cells was not observed.



81

2.9 Enhancement of Bioavailability

2.9.1 Introduction

Advances in drug design have increased the number of 'hits' obtained in a shorter amount of time in comparison to conventional medicinal chemistry using methods such as combinatorial chemistry and high-throughput screening. Despite these advances, many drug candidates fail to negotiate the development process due to inadequate absorption, distribution, metabolism and excretion (ADME) properties. A drug can fail the process *via* one of these qualities or a combination. It has been estimated that approximately 40% of drug candidates fail the clinical trial stage due to poor ADME properties, problematic since late-stage failures contribute significantly to the ever-increasing cost of investigational drug development.[470] The efficacy and kinetics of therapeutically active compounds are a function of their ADME properties, all of which influence the pharmacological effects of the drug by determining the drug concentration within the target and surrounding tissues. Poor ADME properties are the most common reasons for the termination of a drug during development, hence the increasing emphasis on the rigorous pre-assessment of these properties.

When designing a drug molecule, oral activity is the objective all medicinal chemists hope to attain as this is the most beneficial route for both the patient and health worker. However, oral absorption is a multifaceted process surpassing a gauntlet of enzymes with little absorption occurring until the drug enters the small intestine. The efficiency with which the molecule does this is dependent on the molecular constitution of the drug, the physiology of the gastrointestinal tract (gastrointestinal motility, splanchnic blood flow) and the method by which the drug has been formulated.[471] Therefore absorption of drugs from the GI tract is dependent on the ability of the molecule to traverse the intestinal cell membrane. As expected, strong bases of pK_a 10 or greater are poorly absorbed, as are strong acids of pK_a 3 or less since they are fully ionised. There are several clinically important drugs that are strong bases and consequently administered intravenously since they are poorly absorbed from the GI tract,[471,472] PMD being the most relevant illustration of this.

Overall, the mechanism of drug absorption involves passive transfer through cell membranes of the intestines at a rate determined by the ionisation and lipid solubility of the molecules. In addition, active transport carrier mediated across the GI mucosa may be the method by which drugs traverse the membrane.[471] All drugs must undergo this process if they are to arrive at their site of action.

The poor oral bioavailability of PMD is clearly understood by applying these principles, the amidine moiety being protonated readily within the acid milieu of the stomach thus generating an inability to enter the blood stream for uptake by parasitised erythrocytes. We therefore must establish a method to overcome this barrier associated with the oral activity of diamidine and diguanidine based compounds.

2.9.2 A Prodrug Approach

As discussed, the clinical use of diamidine and diguanidine containing drugs is compounded by their poor oral bioavailability furthermore, this property leads to inter-individual differences and pronounced drug accumulation.[302] This issue arises from the highly basic nature of the amidine functionalities through a pKa > 10 (12-13) rendering the functionality cationic at physiological pH causing an inability to traverse the endothelial cell membrane from the acidic environment of the stomach. Furthermore the antimalarial activity of many compounds appears to be related to the basicity of the cationic group with the optimal pKa being > 12.5.[152] In order for a molecule to traverse the intestinal epithelium, the basicity of the cationic group must therefore be reduced prior to uptake by target cells. Masking these cationic moieties by depressing their ability to protonate at physiological pH would therefore provide a feasible route to the enhancement of their oral potency. Clearly, the attachment of a pro-moiety to the amidine nitrogen **82** would lower its ability ionise, similarly addition to the amidine amino group **83** would lessen the availability of the lone pair for stabilisation of the positive charge (dependent on the functionality) thereby decreasing the ionisation potential of the amidine nitrogen as shown in Figure 23. Effectively, this concept entails a prodrug approach.

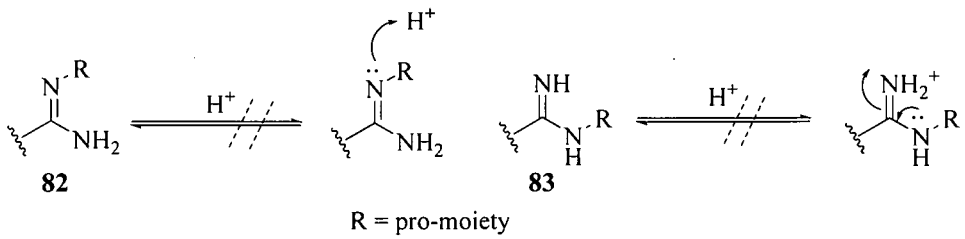


Figure 23.

Studying the literature, the first instance of the term prodrug was in Albert's discussion of the chemical aspects of selective toxicity,[473] defined later as a bioreversible chemical derivative of an active parent drug, besides salts and complexes which may also be regarded as water soluble prodrugs for oral drug delivery.[474] A prodrug approach is delicate to perfect since the prodrug itself must be therapeutically benign yet chemically reactive in order to undergo chemical conversion at a sufficient rate to release the reactive chemical entity such that the molecule can pass the endothelium prior to metabolic cleavage.[471]

Prodrugs are now established as a way of optimising ADME requirements thereby overcoming limitations commonly encountered in oral drug delivery and there are various examples on the market as shown in Figure 24. They have been shown to substantially improve the bioavailability, efficacy, toxicity and formulation of drugs.[270,475]

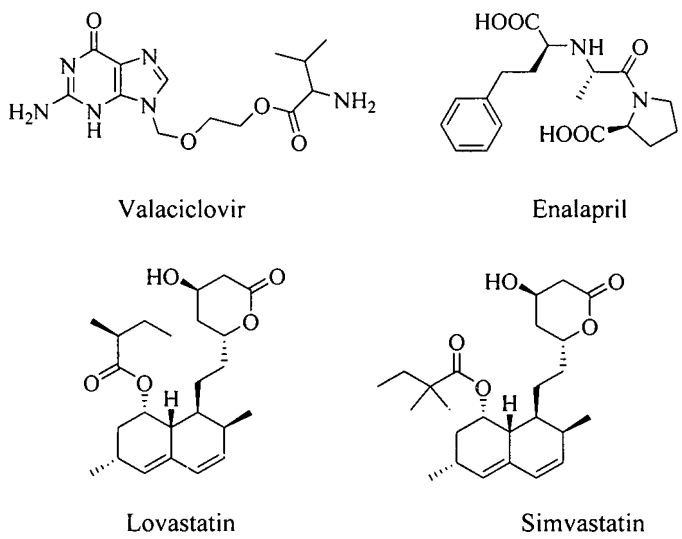
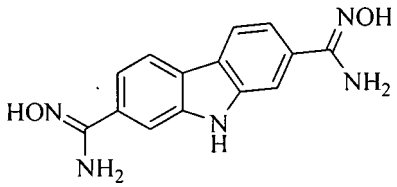


Figure 24.

There are alternative approaches to the improvement of drug delivery aside from the attachment of a pro-moiety for instance, encapsulation in sugar grafted liposomes has been used enhancing the bioavailability and potency of PMD[476] however, most frequently chemical derivitisation is used. *N*-hydroxymethylation and *N*-aminomethylation of amides, imides and urea derivatives as well as *N*-Mannich bases for compounds containing a primary or secondary amino group are possible ways of attaining prodrugs, with *N*-amidomethylation decreasing amine pK_a by 3-4 pK_a units.[475,477] For the amidine functionality in particular, *N*-alkoxy[337,478-480] or *N*-carbamate[478,481] pro-moieties are frequently used.

Several approaches to the development of amidine prodrugs have failed due primarily to poor conversion to the active compound or increased clearance.[337] It has been shown that PMD is rapidly metabolised *in vivo* to at least seven primary metabolites with hydroxylation resulting in a loss of activity.[401] It is this discovery that led to the consideration of amidoximes as prodrugs with *bis*-amidoximes of DB75 being orally active and less toxic than PMD.[482] It has been suggested that diamidoxime prodrugs are effective against extracellular parasites because they enter host cells where they are reduced to the active amidine form, released extracellularly and then taken up by the infecting organism.[480]

It has been suggested that amidoximes are suitable pro-moieties for any pharmacologically active amidine however; through the work of Boykin and Tidwell it is evident that this is not the case for all compounds.[337,347,414,480,482] For instance, linear dications boasting *in vivo* activity superior to DB75 hold significantly reduced oral activity when converted to their methamidoxime prodrugs.[337] The point is further stressed by the fact that aromatic amidoximes **84**, **85**, and **86** tested for anti-pneumocystis activity lacked significant oral and intravenous activity despite their parent drugs possessing potent activity by i.v. injection.[347]



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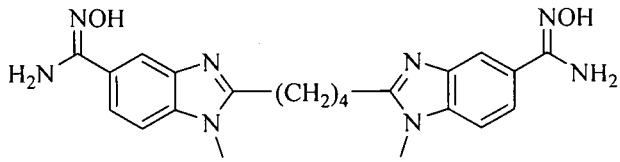

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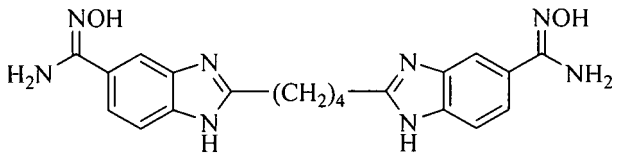
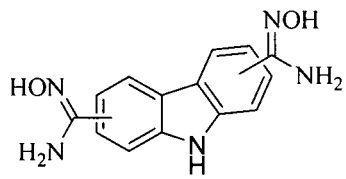
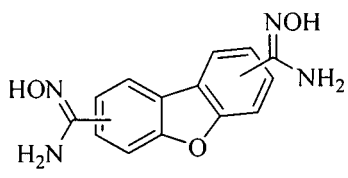


86

This is not an isolated case for this form of fused aromatic system. The poor activity of the diamidoxime carbazoles **87** was presumed to be due to the presence of the carbazole nitrogen therefore dibenzofuran derivatives **88** were synthesised in the hope that the amidoxime dibenzofuran prodrug would be more active. Unfortunately, although the dibenzofuran derivatives displayed potent anti-PCP activity, their amidoximes were less active than the amidoxime of PMD.[482]



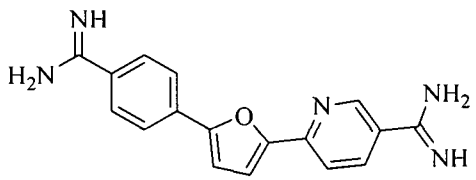
87



88

This lack of activity for particular amidoxime prodrugs has been attributed to inadequate bioconversion to the active moiety and reduced cellular uptake consequently rendering these prodrugs inactive.[337,480] That being said, amidoximes have been observed to possess enhanced oral activity compared to the parent drug[347,480,483-485] though methylamidoximes have shown significantly less toxicity on intravenous dosage than amidoximes.[480] A further illustration of this can be derived from the case of DB820 **89**. DB820 is an aza-analogue of DB75 with potent

activity *in vitro* but poor oral bioavailability. Its methoxy prodrug DB844 **90** has potent activity for both early and late stage African trypanosomiasis, the *O*-methyl and *O*-ethyl analogues again being the most effective.[450] Studies regarding the metabolism of DB844 in human liver microsomes found a dependence on NADPH (nicotinamide adenine dinucleotide phosphate) resulting in the production of over 8 metabolites in 90 minutes of incubation. The parent compound DB280 was the last metabolite formed.[483]



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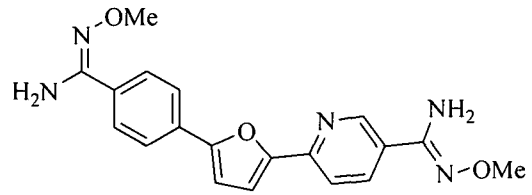


90

2.9.3 DB289

DB75 and its prodrug DB289 were developed by the Boykin and Tidwell groups for the treatment of African trypanosomiasis with prodrug routes initially centering on the development of the amidoxime. The finding that the amidoxime for this compound possessed poor oral activity led to the assessment of *bis*-methyl and -ethyl amidoximes of DB75 with the *O*-methyl amidoxime being the most effective and least toxic of the compounds tested.[480]

DB289 metabolism is believed to undergo an ordered process of *O*-demethylations and dehydroxylations of the amidoxime as depicted in Figure 25, with cytochrome b_5 and b_5 reductase shown to play a direct role in the metabolic activation of DB289 to DB75.[486,487] The prodrug undergoes first-pass metabolism mediated by CYP4F enzymes catalysing the initial oxidative *O*-demethylation of DB289.[488]

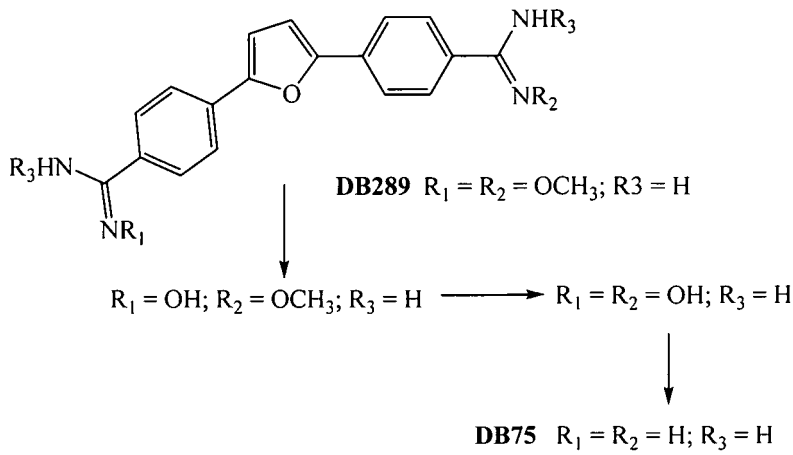


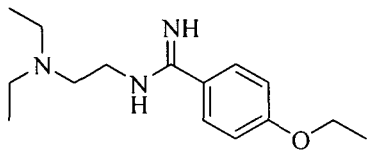
Figure 25.

As discussed, for oral activity there is a requirement for the drug molecule to traverse the epithelium in order for uptake into the intestinal tract. It has been suggested that the increased oral activity of DB289 compared to the parent drug could be due to their varying transport routes.[480] This has been substantiated by studies regarding the transport of DB75 and its prodrug across caco-2 cell monolayers where DB75 transport occurs *via* a paracellular route, however DB289 has a transport rate 85-fold higher than that of DB75 and is transported transcellularly.[485] The implications of which are clear when considering that paracellular transport involving passive diffusion is less efficient than the transcellular route due to a lower surface area available to the molecules entering the intercellular space.[485,489] Distribution studies within trypanosomal systems suggest that DB289 would have limited activity during the late stages of the disease since oral administration of DB289 showed distribution into the brain parenchyma, but concentrations of the active drug were low[490] therefore also having possible implications for use against the cerebral malaria stage.

Within the malarial parasite, DB75 is active *versus P. vivax* 15 fold more than against *P. falciparum* attributed to the slow onset of activity for DB75 and hence a requirement for more than an incomplete blood stage cycle to display its full effect.[491] DB75 and DB289 were assessed for the first time for the treatment of *P. vivax* and acute uncomplicated *P. falciparum* infections in patients at the Hospital for Tropical Diseases, Bangkok for three months. A cure rate of 96 %

was achieved by the oral dosing of DB289 at 100 mg twice a day with parasitemia cleared in most patients by day 7. Patients with *P. vivax* infections were also treated with primaquine on days 10–23. Excitingly, DB289 was well tolerated and any adverse effects observed were mild.[492]

Aside from amidoximes, other prodrug approaches have been used for the masking of the amidine moiety for instance, the acetylation of the antiarrhythmic benzamidine **91** improving oral activity markedly.[493]



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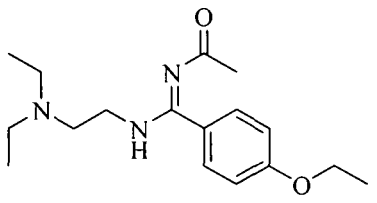
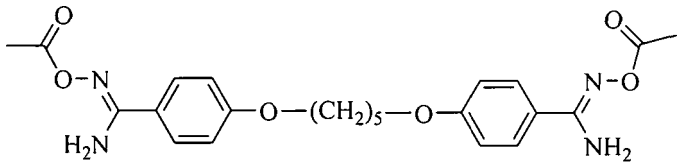


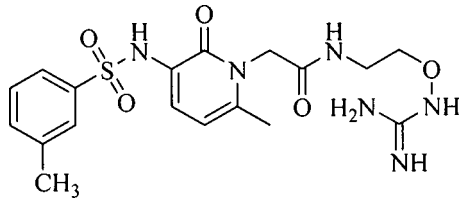
92

In addition, diacetyldiamidoximeesters of PMD **93** have been used as prodrugs enhancing the efficacy of PMD by improving its lipophilicity. The biotransformation to the active drug occurring *via* cleavage of the ester moiety and *N*-reduction.[484]



93

Carbamates have been used as pro-moieties for the amidine unit within selective factor VIIa and thrombin inhibitors **94** and **95** which possess low oral bioavailability. For these compounds, a carbamate prodrug approach yielded little improvement on activity due to poor conversion to the amidine postulated by Riggs *et al.* to be due to the biaryl scaffold not being recognised as a substrate for the enzymes necessary to effect the amidine prodrug conversion.[478,481]



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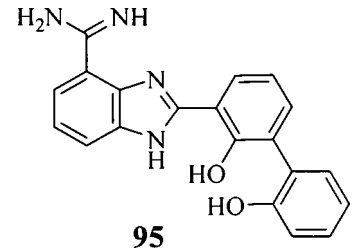
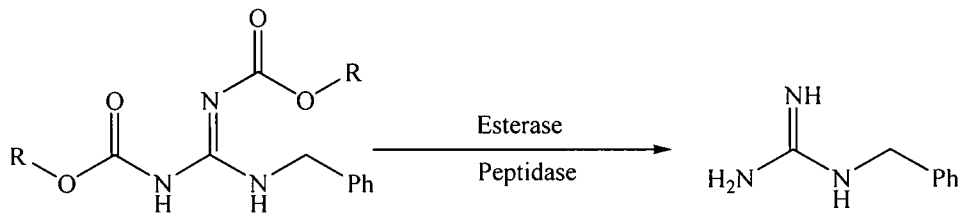


95

2.9.4 Guanidine prodrugs

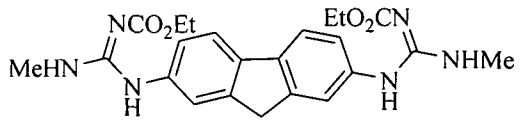
It seems the first example of a pro-moiety for the guanidine functionality originated from the work of Saulnier *et al.* regarding the improvement of the formulation and overall bioavailability of potential guanidine containing drug candidates. Using *N,N'-bis*(acyloxymethoxycarbonyl)-*S*-methylisothioureas and a primary amine they formed compounds **96** and **97**, shown to function as enzyme labile prodrugs for the free "drug" **98**.[494] In addition to compounds **96** and **97** other guanidine prodrugs have been shown to be activated to their parent guanidine containing drugs by esterases.[495-497]



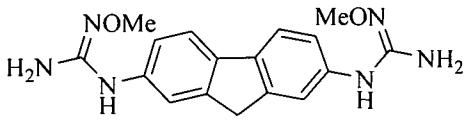
96 ; R = CH_2OCOCH_2Ph
97 ; R= $CH_2OCOC(CH_3)_3$

98

Approaches using *N*-alkoxy and -carbamate pro-moieties have been employed for use within the African trypanosomiasis animal model generating carbamates possessing *in vivo* activity with compound **99** giving a cure rate of 4/4. In contrast, the *N*-alkoxy analogues assessed in this model, **100** and **101**, were ineffective as prodrugs.[224]



99



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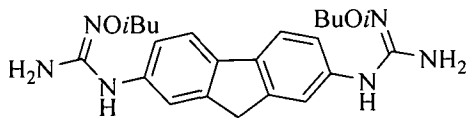
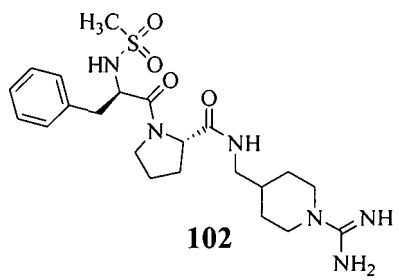


101

The guanidine containing compound **102** is a potent antithrombotic agent in animals when given systemically however its oral activity is poor due to poor intestinal absorption. Prodrug methodology was implemented generating lead acyl prodrugs **103** and **104** with compound **103** giving limited improvement in permeability across caco-2 monolayers while compound **104** produced a marked improvement.[497]



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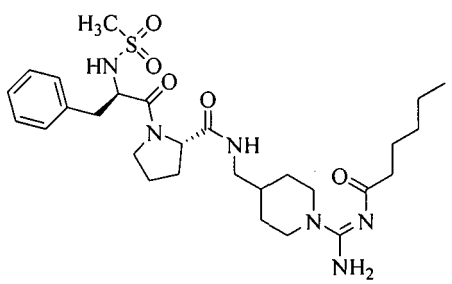

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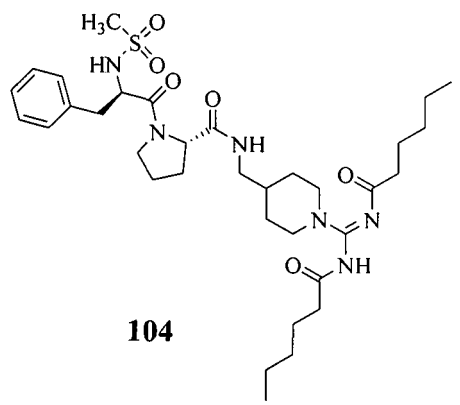
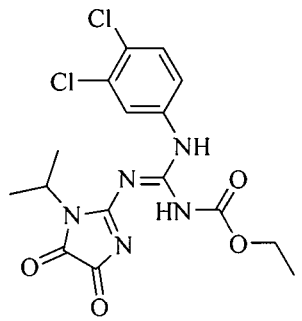


104

Searching the literature, investigations into the study of diguanidine prodrugs within the field of malaria is limited. However, WR182393 is a 2-guanidinoimidazolidinedione mixture with high prophylactic and radical curative antimalarial activity.[498] The development of WR182393 was however compounded by its poor aqueous and organic solubility thus rendering purification of

the mixture and structure identification problematic. Guan and co-workers developed carbamate prodrugs **105** and **106** from the title mixture finding that the solubility of the product was enhanced thereby enabling its separation and purification. After structural determination of the two components within WR182393, further alkyl carbamate analogues were prepared with lead compounds **107** and **108** possessing an increased intramuscular efficacy than WR182393 against *Plasmodium cynomolgi* in the Rhesus monkey.[495]



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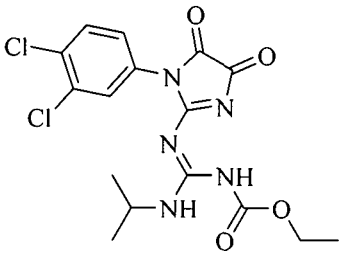

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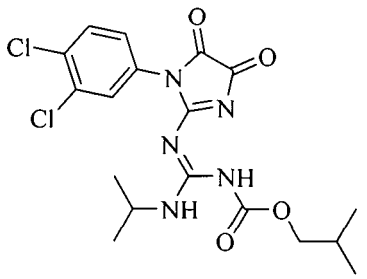

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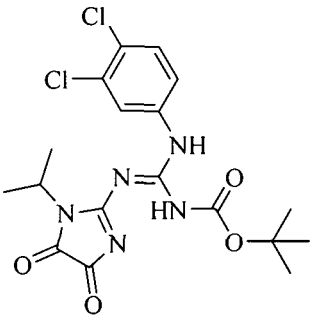


108

2.10 Summary

In what can only be described as an escalating battle, malaria has proliferated to epidemic levels thus the development of novel drug templates is vital, particularly drugs that are parasite specific with moieties unrecognisable by the parasite. Evidently, amidine and guanidine containing drugs are a promising class of anti-infective agents. They have a broad spectrum of activity inhibiting a range of cellular processes in various organisms furthermore there are amidine and guanidine containing compounds possessing potent antimalarial activity.

The deployment of these drugs is however plagued by issues surrounding their poor oral bioavailability, though advances in prodrug design facilitate a route to their oral use. Host cell toxicity to these molecules is frequently reported in the field, a factor connected to their route of administration and poor absorption. That being said, the most notable diamidine pentamidine has been used clinically for the treatment of trypanosomiasis for over 50 years.
Further studies are required to ascertain their mode of action in the malaria parasite, especially regarding their DNA binding properties and possible interactions with the *bc1* complex since as yet, the role these properties play remain unknown.

As antimalarials, diamidines are fast becoming a subclass in their own right, their future as anti-infective agents firmly grounded in the applicability of prodrug approaches.

CHAPTER II

PART II

Fluorene

The aromatic hydrocarbon fluorene forms the template used for the generation of *mono-* and bis-cationic antimalarials and hence the structure, chemistry and medicinal chemistry of the fluorene unit and fluorene containing compounds is reviewed here. This chapter will contain a separate numbering system for all compounds, figures and schemes.

2.11 An Introduction to Polycyclic Aromatic Hydrocarbons and Fluorene

Polycyclic aromatic hydrocarbons (PAHs) are molecules consisting of two or more fused aromatic rings, the simplest of which is naphthalene. PAHs are not essential for the growth of living cells and are generated as a by-product of fossil fuel combustion, and in fact are the most widespread pollutants. The majority of PAHs are linked to ill heath effects, the most notable being genotoxicity.[499,500] For many years the scientific community has recognised that exposure to coal tar induces tumours which do not correlate with the benzopyrene content and are due to PAH fractions. Bioactivation to reactive intermediates that bind to DNA is widely accepted to be the mechanism by which this happens since PAHs are enzyme inducers, substrates and inhibitors.[501,502] The ability of fluorene (PAH, Figure 1) and fluorene containing compounds to form adducts with DNA has been demonstrated[503,504] by Koganti *et al.* who observed that a hydrocarbon with a mass greater than 216 was responsible for the formation of a chemical-DNA adduct. Also noted is the fact that the toxicity of PAHs generally decreases with increasing molecular weight,[505] presumably because absorption is less efficient.

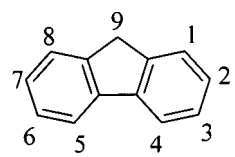


Figure 1. Structure and numbering system of 9*H*-fluorene

Fluorene is a crystalline solid found in various natural products and petrol engine exhaust gas, the molecular structure and numbering system is shown in Figure 1. The name fluorene is attributed to the bright violet fluorescence discharged under UV light. The fluorene unit has found widespread use due to its properties including; conductivity,[506,507] emittability,[508-510] light and temperature sensitivity/ stability,[509,511] heat resistance[512-514] and resistance to corrosion.[515] Insoluble in water, fluorene is however soluble in most organic solvents; isopropanol (IPA),

ethanol (EtOH), *n*-butanol, dimethylbenzene, methylbenzene, benzene, acetone and ether. Applications of fluorenes are highly varied for instance pharmaceutical and medicinal chemistry research,[516-519] plastics, dyes,[509] pigments (optical brightening agent),[514] pesticides, synthetic resins,[520] organic synthesis (protecting groups,[521,522] ligands in metallocene catalysis,[523] organolanthanides[524]), LEDs,[525-527] microscopy[509] and as a building block for the generation of dendrimers,[528-530] polymers[531,532] and oligomers.[533-535] They are used in the applications of thermo and light sensitizers, liquid crystal chemistry, luminescence chemistry, spectrophotometric analysis, molecular chemistry, and organometallic complexes. More recently fluorenes are being assessed as a component for solar cells.[536]

2.12 The Structure of Fluorene – Uniplanar *vs.* Folded

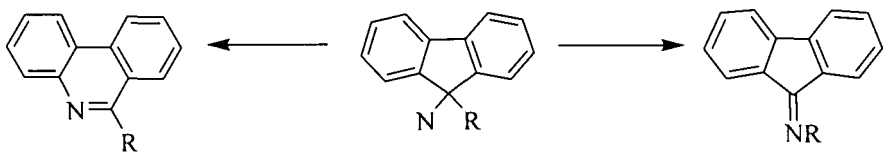
The structure of fluorene has caused much debate,[537,538] with the main argument regarding whether this seemingly simple molecule is uniplanar or folded with the planes of the six membered rings at an angle to the plane of the central ring. In 1925, a structure for fluorene (Figure 2) was generated to explain the separation of two forms of 9-aminofluorene although attempts to repeat this separation have been unsuccessful or disproved.[538,539]

Figure 2. The two structures of fluorene proposed by Kuhn and Jacob.[539]

Ten years later, Pinck and Hilbert commenced x-ray crystallographic studies and noted that the benzene rings adjacent to the central five membered ring must be distorted by 12° or 13° in order for the bond distances obtained to be correct as depicted in Figure 3. If this were indeed the case then the rigidity of the phenyl rings would indicate that the central ring would be under strain to distort to this degree. This proposal can be questioned since the calculations are based on a single fixed bond structure.[537,538]

Figure 3. Pinck and Hilbert's distortion of the valence angles from the benzene rings by 12-13° gives a slightly bent structure.[538]

Pinck used the degree of strain argument to add to speculation that fluorene was indeed non-planar by means of the premise, that if the molecule was under a degree of strain then it would be readily open to ring expansion. Pinck and Hilbert applied the Stieglitz rearrangement for the synthesis of a phenanthridine derivative, the idea being that strain would be evident by the ease of ring expansion of the 9-substituted fluoryl nitrogen. They established that rather than forming imide **2** *via* migration of the R group, the six membered ring **1** was generated *via* ring expansion thus concluding that ease of strain of the five membered ring was an important molecular feature.



1 **2**

It seems however, that Pinck was himself mystified by the nature of fluorene noting that Stuart's interpretation of Hengstengerg's 1929 x-ray data favours a planar structure.[540] It appears that most of the debate surrounding the structure of fluorene arose from the method of analysis and quality of data obtained. X-ray crystallographic data of Cook and Iball supports Pinck's folded molecule, although the angle at which this distortion occurs differs from Pinck (12-13°) with Iball stating it to be 20°.[541] The x-ray analysis of fluorene was also undertaken by Brown in 1954, the results of which correlate with Iballs 1936 structure.[542] The x-ray studies undertaken were of a qualitative mode and consequently did not give comprehensive data for interpretation.

In 1954, Burns completed a detailed quantitative analysis of the fluorene crystal complete with bond lengths (mean bond length in benzene ring = 1.403 Å) concluding that the structure must be planar.[543] Further evidence for the elucidation of the fluorene structure, can be obtained from resonance energies and bond characters of the relevant carbon-carbon bonds since if the structure was indeed folded, resonance would occur through the benzene rings only, however this is not the case. In addition, the resonance energy of fluorene is larger than that of two benzene molecules alone (101 *vs.* 82 Kcal/mole).[544]

Non-planarity across the structure would have its consequences since the asymmetry induced would lead to the possibility of isomer formation *via* substitution at the 9 position *cis* or *trans* to each ring. Therefore if two isomers could be separated this would add weight to the non-planar argument. Examining the literature, it is clear that the resolution of fluorene isomers was problematic since there are few successful reports. Ray and Kreiser did successfully resolve 9-hydroxyfluorene-2-carboxylic acid and 9-aminofluorene-2-carboxylate into its *dextro* and *levo* forms using strychnine and *d*-tartaric acid respectively.[545] Weisburger *et al.* were less successful, unable to resolve a *mono*-substituted spirobifluorene leading them to conclude that fluorene is planar.[544]

As illustrated by this discussion, the evidence for the structure of fluorene differs based on the data presented. Dipole measurements, x-ray studies and absorption data suggest a flat planar arrangement since the spectrum is similar to that of biphenyl[544] whereas ring enlargement can be interpreted as alleviation of ring strain due to bent biphenyls.[537] Post 1960, literature concerning fluorene refers to the fluorene structure as being planar.[546-549] However, studies have shown that substitution at the 2 and 9 positions produces compounds with crystal structures that consist of planar molecules symmetrically placed with their planes inclined at 28°[549] and 55°[550] respectively. In addition, carbanions of fluorene have been shown to have both fused phenyl rings planar with the 5-membered ring. The α-carbon is also sp^2 hybridised and therefore the negative charge can resonate into both planar aromatic rings.[551] Zerger *et al.* obtained the molecular and crystal structure of fluorenylpotassium and fluorenyllithium from single crystal X-ray data, which correlates with Gerkin's findings that the fluorenyl carbanion deviates from uniplanarity. Both phenyl rings were found in the same plane with carbon 9 pulled out of the

plane of one benzene ring towards the lithium cation by 0.042 Å.[552] Moreover the analysis previously obtained by Burns and Iball, which led them to conclude a planar structure, did not account for or report any disorder in the fluorene crystal. Gerkin *et al* found that the entire fluorene unit is not planar. The asymmetric unit bisecting C (1) to C (7) is planar and this gives the molecule the 'V' shape depicted in Figure 4.[553]

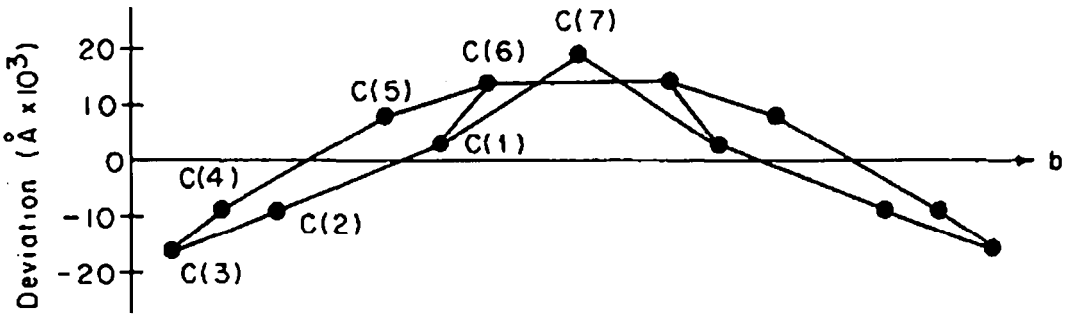


Figure 4. Deviation of the C-atom positions from the mean molecular plane of the fluorene molecule gives a fluorene a 'V' shape.

The disparity concerning whether fluorene is uniplanar or not is still unresolved, with Belsky[554] and Gerkin[553] in disagreement some 40 years after the work of Pinck and Hilbert.[537] There is a 0.019 Å difference in bond lengths obtained, which may be due to an alternate conformation of the molecule at different temperatures. However, Gerkin *et al.* performed their calculations at both 295 and 159 K finding that fluorene is planar at the asymmetric unit only, whereas Belsky *et al.* establish uniplanarity in the molecule at 295 K only. The symmetry of fluorene is on the other hand in agreement as being C_{2v}[553-555] as is the nature of the intermolecular bonding being entirely Van der Waals based.[549,550,554] The discrepancy regarding the structure of fluorene is difficult to access since the literature is absent of a definitive answer. Nevertheless, we have used density functional theory in order to access the data in-house leading to the structure depicted in Figure 5. Based on our calculations (using density functional theory B3LYP/6-31G) we established a uniplanar fluorene structure.

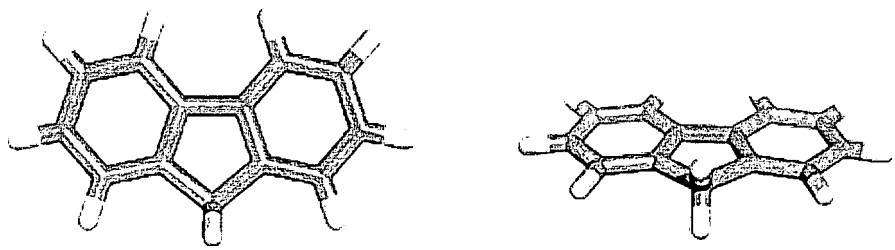
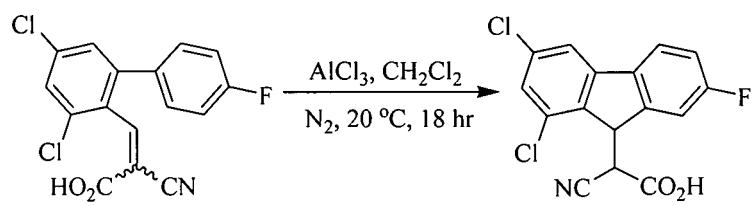


Figure 5. In-house representation of the structure of fluorene.

2.13 Fluorene Preparation

Most PAHs are not synthesised chemically but are isolated from natural sources such as oils and hydrocarbons usually by pyrolysis followed by subsequent purification through repeated distillation and crystallization.[556] Fluorene itself is a major component of anthracene oil[557] and coal tar where it can be isolated from the middle oil fraction or distillation of fluorenedicarboxylic acid with lime.[558]

The fluorene unit can also be constructed *via* synthetic methods in order to generate fluorene analogues. Stokker used aluminium chloride to induce an intramolecular Friedel-Crafts alkylation of the 2-cyanoacrilic acid precursor (Scheme 1) forming the product, 2-cyano-2-(1,3-dichloro-7-fluoro-9H-fluoren-9-yl)acetic acid **4** in moderate yield (60 %).[559]

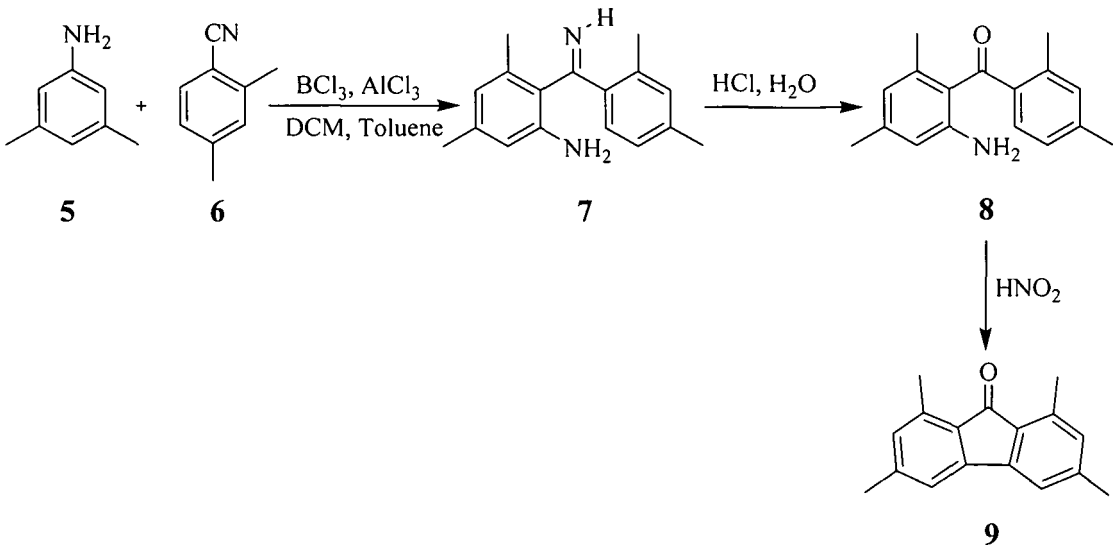


3 **4**

Scheme 1. Friedel-Crafts alkylation of 2-cyano-3-(3,5-dichloro-4'-fluorobiphenyl-2-yl)acrylic acid

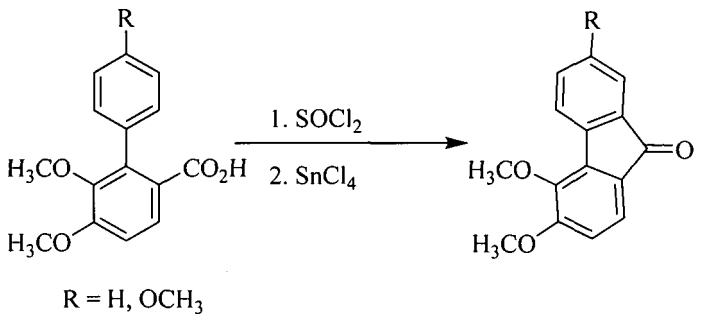
They again performed ring closure in order to prepare several symmetrical fluorenone derivatives from aniline and the nitrile precursor. Pschorr ring closure of benzophenone **8** was

achieved by generating nitrous acid *in situ* by mixing aminobenzophenone and hot concentrated sulphuric acid; quenching with sodium nitrite provides fluorenone **9** in a 43 % yield (Scheme 2).



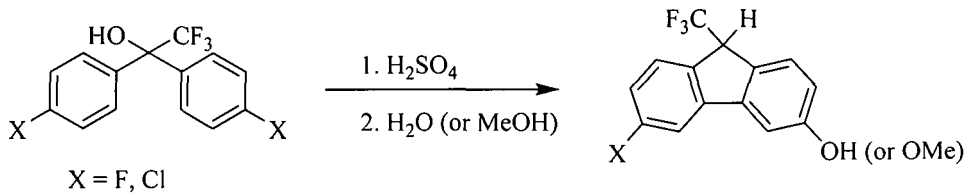
Scheme 2. Synthesis of 1,3,6,8-tetramethyl-9*H*-fluoren-9-one by Stokker *et al.*[559]

Acid chlorides can also facilitate ring closure to give the fluorene unit. Ladd *et al.* used a two-step procedure through sulfonyl chloride and stannic chloride to form the fluorenone shown in Scheme 3. This reaction can also be achieved in one step using thionyl chloride though the yield of the reaction is compromised (94 *vs.* 30 %).[560]



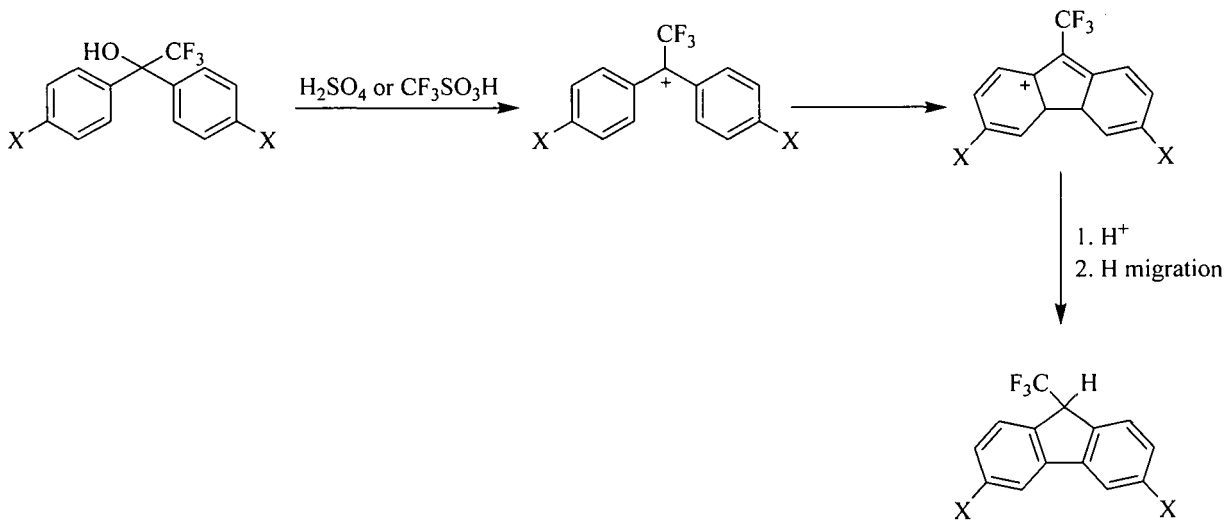
Scheme 3. Synthesis of substituted fluoren-9-ones by Ladd *et* al.[560]

It has also been shown that cyclodehydration of *p*-fluoro and *p*-chloro substituted alcohols mediated by H_2SO_4 and water or MeOH forms the corresponding fluorenes represented in Scheme 4.[561-563]



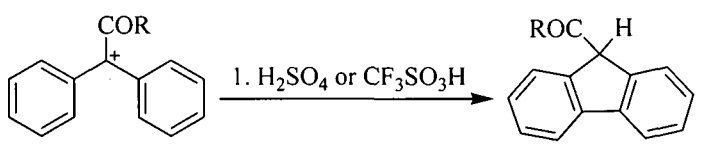
Scheme 4. Cyclodehydration of *p*-fluoro and *p*-chloro substituted alcohols

The mechanism of reaction involves the formation of an unstable cation *via* protonation of the hydroxyl group under acidic conditions. Subsequent loss of water followed by ring closure, protonation and hydrogen migration restores aromaticity.



Scheme 5. Mechanism of reaction of cyclodehydration of *p*-fluoro and *p*-chloro substituted alcohols[563]

Since the methylene carbon of the diphenyl starting material can be functionalised, this permits the construction of other analogues such as the acid, ester, and amide.



Scheme 6. Cyclodehydration of diphenyl analogues

2.14 Brief Review of the Reactivity of Fluorene

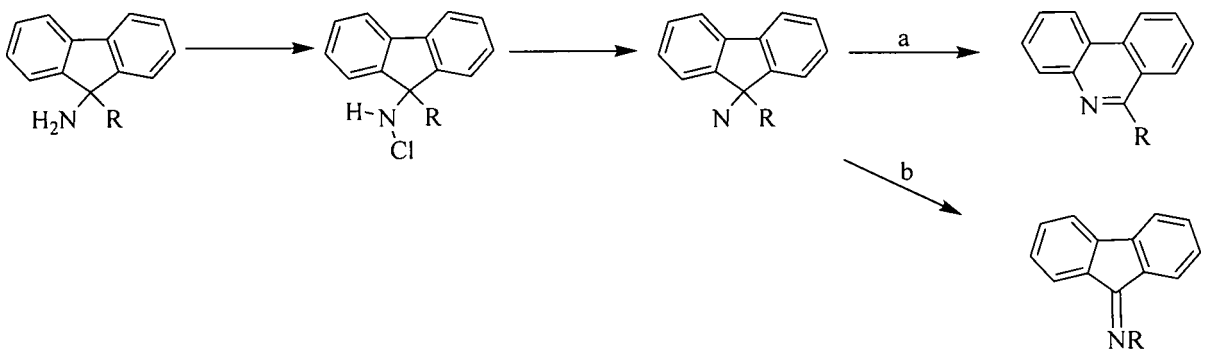
2.14.1 Introduction

PAHs are known to react with molecular oxygen over 200°C to form oxi and peroxi radicals by molecular condensation yielding polymeric molecules with a non-planar structure. These compounds do not incorporate oxygen into their structure removing most of it as water, a process exploited in the isolation of PAHs.[557,564] Most studies concerning the reactivity of fluorene have employed a comparison to biphenyl.[547,548] It is noted that fluorene is more reactive than biphenyl, due to the fact that the biphenyl system deviates from planarity.[565]

Fluorene is a weak acid with five different positions that are susceptible to nucleophilic attack; the phenyl rings and the C-9 position. The protons at C-9 are particularly labile due to their increased ionisability[566] with a pK_a of 25[567] which changes to approximately 9 in the singlet excited state[568] thus enabling rapid ionization. Deprotonation of fluorene is therefore facile and can be achieved easily using mixtures such as, dimethyl sulphoxide-methanol-sodium methoxide or alcohols-sodium alkoxides[569] and alky lithiums in the appropriate solvent.[570] The chemistry of the methylene hydrogens is analogous to cyclopentadiene (Cp). The driving force for anion formation at C-9 is the increase in aromatic character attained, since anionic fluorene has a 6π internal cyclic array thereby imparting greater stability of the anion by satisfying Huckel's rule requiring $(4n+2)$ π electrons, where n is an integer. Photochemically the reactivity of fluorene is somewhat different. Fluorene does not undergo deprotonation upon photochemical excitation[571] even though this would generate a $4n+2$ ground-state anion.

2.14.2 Ring Expansion

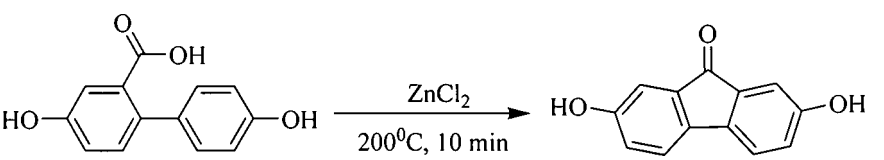
As depicted in Scheme 7, substitution of fluorene at the C-9 position enables the formation of phenanthridine derivatives as the sole product through ring expansion, group migration to form the imide does not occur.[537] The nature of the R groups which can undergo this transformation is non-specific ranging from aryl, alkyl and fluorylamines.



Scheme 7. The synthesis of Phenanthridine derivatives.[537] Route a; ring expansion to give the Phenanthridine. Route b; R group migration to form the imide product.

2.14.3 Oxidation

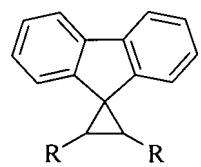
Fluorenone can be obtained naturally as a constituent of coal tar or synthesised by the oxidation of fluorene with reagents such as chromic acid,[572] sodium dichromate[573-576] peracetic acid,[577] nickel peroxide,[578] hot potassium permanganate,[579] silver acetate in pyridine[566] and molecular oxygen in the presence of Triton B and pyridine.[575] Examples of the use of fluoreneone *en route* to products of chemotherapeutic interest are vast.[573,574,577,579-582] Agrawal used fluoreneone derivatives as part of his route to the development of compounds with antitumor activity. He synthesised 2,7-dihydroxyfluorenone by the dehydration of 4,4'-dihydroxybiphenyl-2-carboxylic acid mediated by zinc chloride[580] as shown in Scheme 8.



Scheme 8. Dehydration of 4,4'-dihydroxybiphenyl-2-carboxylic acid mediated by zinc chloride

Fluorenone is widely used as part of the synthetic chemist's arsenal[581,583-589] since it enables the generation of a diverse range of compounds such as phenanthridines,[588] conjugated oligomers,[590] monomers for the synthesis of highly functionalised polymers and copolymers,[584,587] benzimidazoles and spiro-thiazolidinone derivatives[585] and accordingly its reactions will be examined.

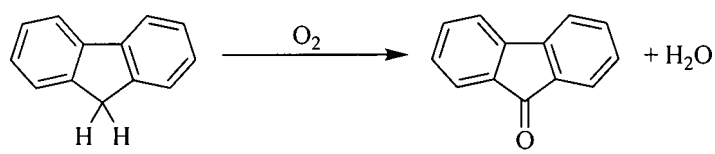
Fluoreneone undergoes the Wittig reaction to give a substituted methylenetriphenylphosphine which when reacted with a second molecule of the phosphorous compound gives spirobicyclics such as **10** (Figure 6). When benzyldiphenyl- phosphinoxide is used with potassium *tert*-butoxide a benzylidiene fluorene is formed.[591]



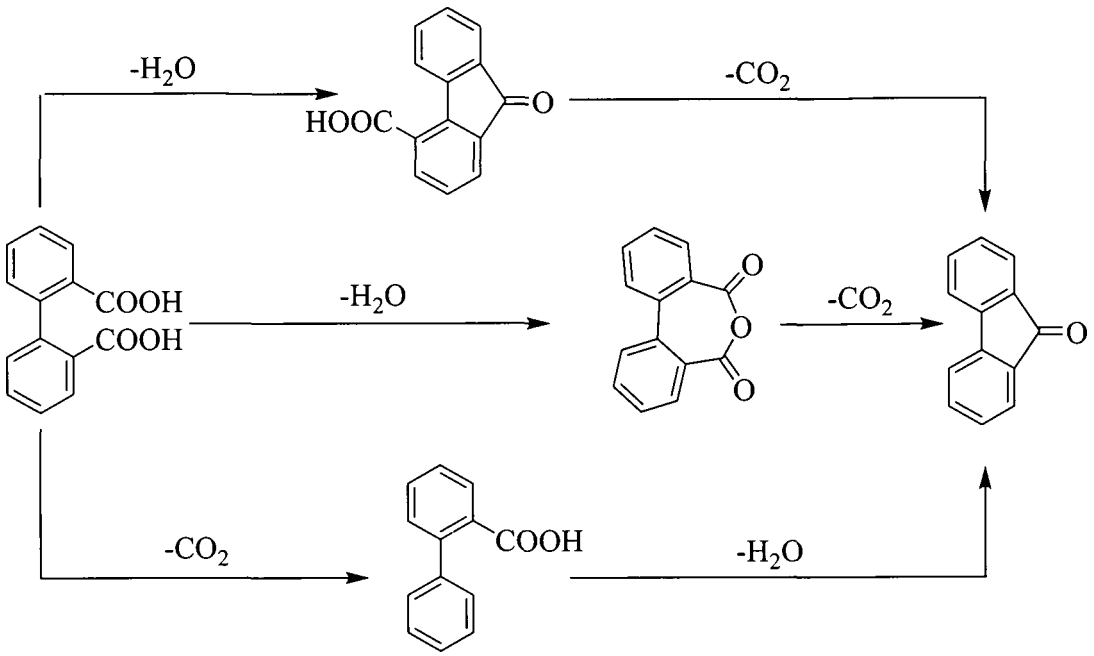
10

Figure 6. Spirans generated from the Wittig reaction.

Reduction of fluorenone with reagents such as aluminium *iso*-propoxide,[588] hydrazine hydrate[574] and sodium borohydride[592] confers a point of diversity, one which was exploited by Arcus in his studies of organic azides and their reactions.[593] Reduction of fluorenones with sodium borohydride was found by Parry to be inhibited by electron releasing groups and accelerated by electron withdrawing groups when in the 2 position.[592] Fluorenone can also undergo alkylation with alkyl, allyl, and phenyl magnesium bromides to yield the 9 substituted fluorylidene product.[544,591] The ionic autoxidation of fluorene in an oxygen atmosphere is achieved by stirring fluorene in pyridine at room temperature with a small quantity of Triton B^{TM}. Heat and water are generated as by products with the reaction proceeding through an ionic mechanism.[566]



Diphenic acid can also be used for the synthesis of fluorenone. The acid can be synthesised from 2-(chlorodiazenyl)benzoic acid[594,595] and heated to evolve carbon dioxide and form quantitative yields of fluoreneone (Scheme 9).[596] The reaction is robust and does not require the use of pure dipenic acid or indeed diphenic acid alone since fluorenone-4-carboxylic acid and diphenic anhydride have been used to the same effect producing only trace amounts of the diphenyl product.[596]



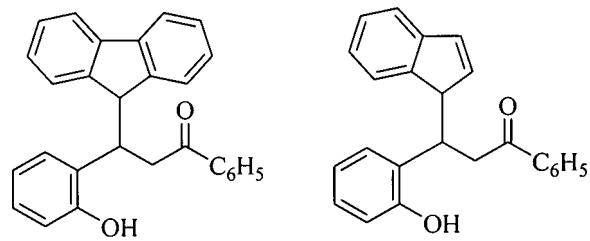
Scheme 9. The preparation of fluorenone from diphenic acid.[596]

Fluorenone carboxylic acid can also be formed from the oxidation of fluoranthene (component of carbon black and coal tars) with potassium dichromate in sulphuric acid. The carboxylic acid can then be converted to fluorenone by distillation.[597]

2.14.4 Reactions at the 9-Position

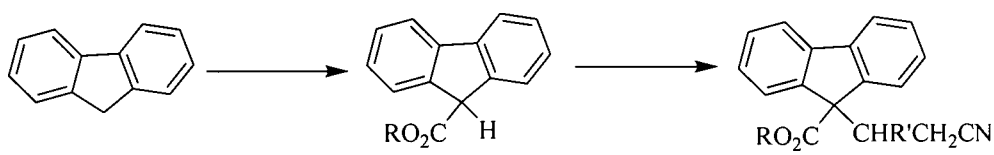
The hydrogen atoms at the 9 position are labile and susceptible to electrophiles. The anion of fluorene can be prepared at room temperature in a variety of solvents by bringing a solution of the hydrocarbon into contact with an alkali metal,[598] although it is noted that 9-fluorenyl-sodium can be difficult to work with.[599] Due to the nature of its absorption spectrum, the fluorenyl anion has received much attention.[598,600,601] The counterion, solvent and temperature used all exert an effect on the anion which has been understood by Hogenesc to be due to an equilibrium between contact and solvent-separated ion pairs.[600] Acrylonitrile reacts in a Michael fashion with fluorene to give the dicyanoethylated product in the presence of a base.[602] Examples of bases employed for this reaction include sodium or potassium methylate, potassium or sodium hydroxide and Triton B™. The *mono* cyanoethylated product can also be formed by using crotononitrile as the

Michael acceptor. In the presence of potassium hydroxide, indene and fluorene undergo a Michael reaction with salicylideneacetophenone to give a mixture of products **11** and **12**.[591]



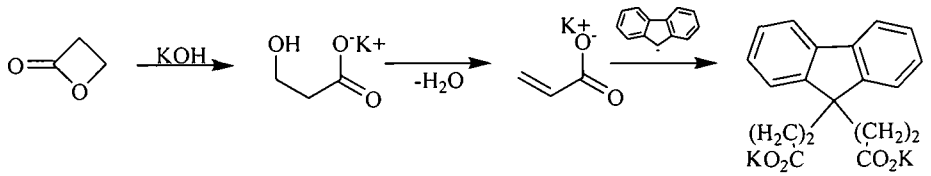
11 **12**

The hydrogens at the 9 position can be made more reactive by esterification of one hydrogen to form the carboxylate (Scheme 10). This enhances the activity of the remaining hydrogen thereby facilitating the addition of the chosen allyl cyanide.[603]



Scheme 10. Michael addition of crotononitrile to fluorene-9-carboxylate.[603]

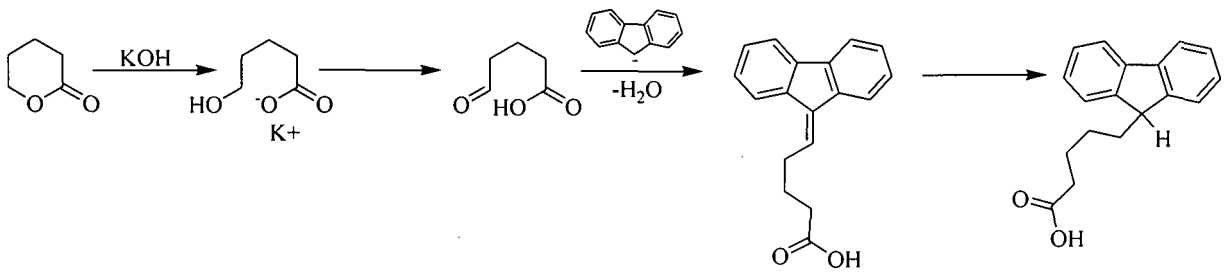
The base catalysed alkylation of fluorene with alcohols or hydroxy acid salts gives 9-alkylfluorenes and 9-fluorenylalkanoic acids respectively in good yields.[567] 9-Fluorenylalkanoic acids can be generated by the addition of a strong base (for example potassium hydroxide) to a lactone at 200-220°C generating the hydroxy acid salt *in situ*. Fritz found that when hydroxy acid salts of lactones including butyrolactone, valerolactone and caprolactone are used, the reaction proceeds to give the *mono* substituted product (Scheme 12). However, when the contributing lactone is propiolactone a *bis*-substituted product is formed (Scheme 11). Fritz postulates that since acrylate esters are known to condense with activated methylene groups, the formation of the *bis*-substituted product could be explained by ring opening of propiolactone in the usual fashion to give a hydroxy acid salt which dehydrates to form an acrylate. This can react again *via* Michael addition to give the *bis*-substituted product **13**.



13

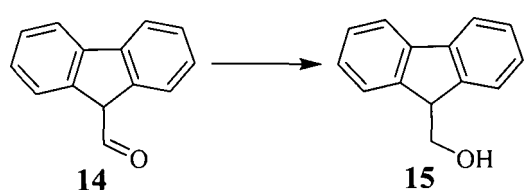
Scheme 11. The proposed mechanism for the reaction of fluorene and propiolactone to form a *bis*-substituted product.

9-Substituted fluorenes cannot undergo reactions of this type since the reaction requires both hydrogen's in the 9 position to form an alkylidene intermediate. The mechanism of reaction for most lactones involves oxidation of the alcohol function of the hydroxyl acid salt, to form a carbonyl moiety which condenses with the fluorenyl anion to form an alkylidene intermediate which is reduced, either by hydrogen present from the dehydrogenation of alcohol or by an alkoxide to yield the 9-fluorenyl pentanoic acid product (Scheme 12).



Scheme 12. The proposed mechanism for the reaction of fluorene and lactones. Various lactones have been used[567], δ-valerolactone is used here.

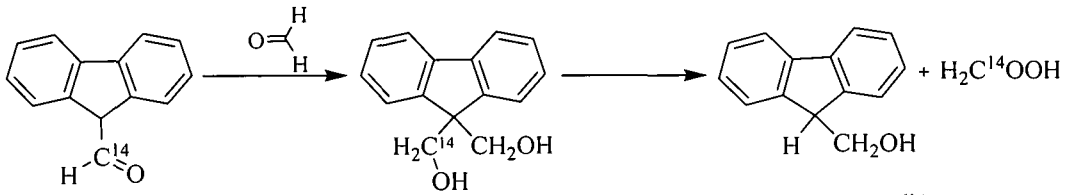
The popularity of the protecting group Fmoc (9-fluorenylmethyloxycarbonyl) for structures containing an amino functionality led to the requirement for a convenient large scale synthetic route to the key precursor of all Fmoc derivatives ((9-fluorenyl)methanol) (**15**) (Scheme 13).[604]



14 **15**

Scheme 13. The key precursor (9-fluorenyl)methanol (15) is synthesised from aldehyde (14).

The aldehyde precursor is obtained by condensing fluorene and ethyl formate in ether using sodium ethoxide as the condensing agent.[560,605] When potassium ethoxide was used the quantity of aldehyde formed was increased due to the improved stability of the aldehyde upon storage. (9-Fluorenyl)methanol was then generated using aqueous formaldehyde in 10 % sodium hydroxide solution. The mechanism of reaction was shown, by radiolabelled ^{14}C, to involve methylation of the hydroxyl group followed by deformylation catalysed by the base (Scheme 14). Unreacted formaldehyde was not radioactive thus ruling out exchange between formylfluorene and formaldehyde, or between formate and formaldehyde.[606]

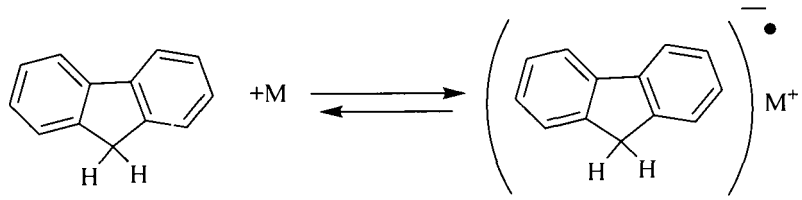


Scheme 14. Mechanism of reaction of 9-Formylfluorene-C^{14} with formaldehyde[606]

Carpino attempted the preceding method for the production of (9-fluorenyl)methanol finding the procedure tedious since a significant number of polymeric bi-products were produced which were difficult to separate from the desired material, therefore impacting the yield of reaction.[604,607,608] Ten years after trying this method, Carpino solved these difficulties by using sodium borohydride in place of formaldehyde for the conversion of **14** to **15**.[604] In addition to increasing the yield of the reaction from 50-60 to 80 %, the purity of the product increased markedly, so much so that it can be used directly from the reaction mixture. More recently, Chong *et al.* synthesised 9-fluorenylmethanol from fluorene using butyl lithium and paraformaldehyde[609] generating the required product in a yield of 73 % avoiding the formation of 9-fluorene carboxaldehyde which is both unstable and a lachrymator. A small amount of the diol was formed, eliminated by recrystallisation from a mixture of hexane and ethanol. The choice of solvent, base and stoichiometry used is critical since when performed in conditions other than 1 equivalent of BuLi and paraformaldehyde in THF only trace amounts of fluorenylmethanol or increased amounts of the diol were isolated.

2.15 Metallation

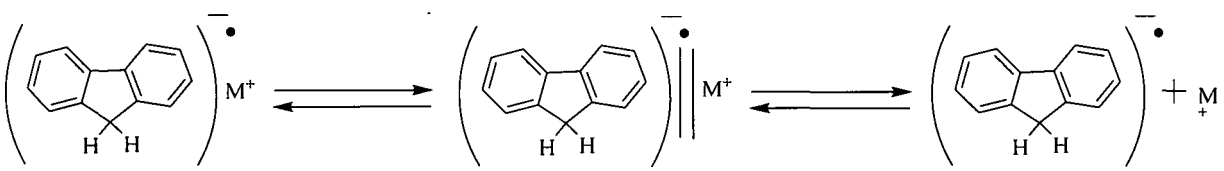
Conversion of the parent hydrocarbon to metallo-hydrocarbon adducts is widely used to functionalise the parent structure.[610-616] When fluorene is reacted with alkali metals such as lithium at room temperature in aprotic solvents for instance THF, a yellow solution of the fluorene anion is produced.[598,601] If the temperature is lowered to -70°C, a stable blue solution of the fluorene radical anion **17** (Scheme 15) is formed which decays to the previous yellow colour when the temperature is increased. The rate of which depends on the nature of the counter ion, solvent and temperature.



16 **17**

Scheme 15. Formation of a fluorene radical anion at -70°C where M represents the alkali metal

The rate is not the only factor that is dependent on these factors. By studying the electronic absorption spectra of the fluorenyl anion, Casson[598,601] recorded three types of ion pair in equilibrium (Scheme 16), contact **18**, solvent separated **19** and dissociated **20**. The equilibrium transfers from contact to solvent separated with increasing solvent polarity, decreasing temperature and size of the cation.[617]



"contact" "solvent separated" "dissociated"

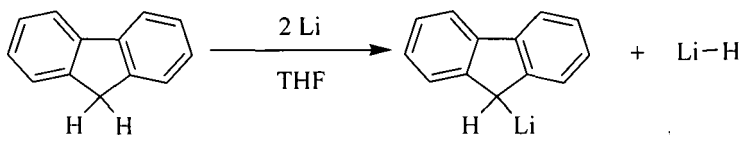
18 **19** **20**

Scheme 16. The nature of the fluorene radical ion pairings present in solution[601]

Most ion pairs of the fluorenyl-lithium salt are solvent separated at room temperature.[600] The decay of the radical anion is believed to be dependent on the concentration of neutral

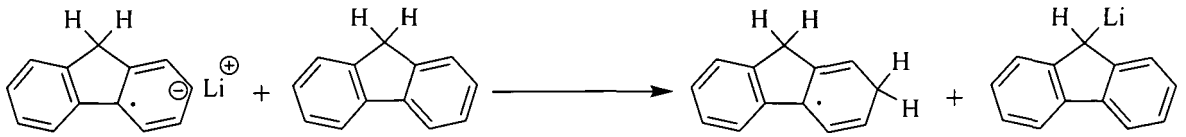
hydrocarbon,[618] being stable if no unreacted hydrocarbon is present in solution.[619] Stability is also dependent on the nature of the cation. In the case of sodium, the kinetics for radical-anion decay are the most complex and difficult to analyse.[620] Lithium is the most stable counterion of the alkali metals in the order Li > Na > K.[619,621] For substituted fluorenes the nature of the substituent markedly affects the rate of decay in the order –OMe> -CN> -Me > -H > -NO_2 when substituted at the 2 position.[621] Waack found dianions of the antiaromatic compounds biphenylene and anthracene, leading to the credence that fluorene may also form dianions although there is little evidence of this.[622]

Cp and indene react with alkali metals to form an organometallic compound and hydrogen evolution. Consequently the lithiation of fluorene was assumed to proceed in a manner akin to its more acidic counterparts as shown in Scheme 17. Quantitative monitoring of lithium consumption found that lithium hydride was not produced, nor was the evolution of hydrogen gas.[623]



Scheme 17. Proposed mechanism of metallation of fluorene with lithium in THF[623]

When lithium metal reacts with fluorene, a dark green precipitate is observed on the metal surface. Hence, the mechanism is believed to involve initial formation of an ionic fluorene-lithium species **21** (responsible for the precipitate), which deprotonates a fluorenyl phenyl ring after which the radical anion **23** is generated. Subsequent quenching of the anion is realised by accommodation of an electron from another unreacted fluorene molecule (Scheme 18). The mechanism of fluorene lithiation is analogous to biphenyl aided by the fact that fluorene is able to form kinetically stable radical anion bases. Hence the reason a small amount of tetrahydro- and hexahydrofluorene forms.[623]



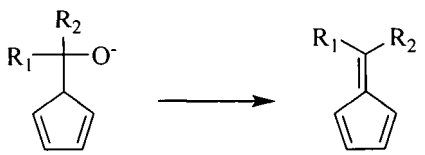
21 **22** **23** **24**

Scheme 18. Mechanism of lithiation of fluorene with lithium metal in THF[623]

The observed rate of reaction regarding fluorene with alkali metals is in the order K>Li>Na.[618] Greenhow *et al.* condensed 9-fluorenylsodium with various alkylating agents including methyl iodide or sulphate, ethyl bromide or sulphate, allyl chloride, benzyl chloride and ethylene dichloride forming the appropriate 9-substituted fluorene.[599] As previously mentioned, the chemistry at C9 is analogous to Cp (**25**) and as such the reactivity of fluorenyl anions with carbonyl compounds can be compared to that of the anions of Cp.

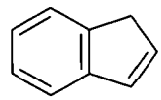
25

Cp reacts with carbonyl compounds to form anion **26** which stabilises itself by fulvene formation (**27**).



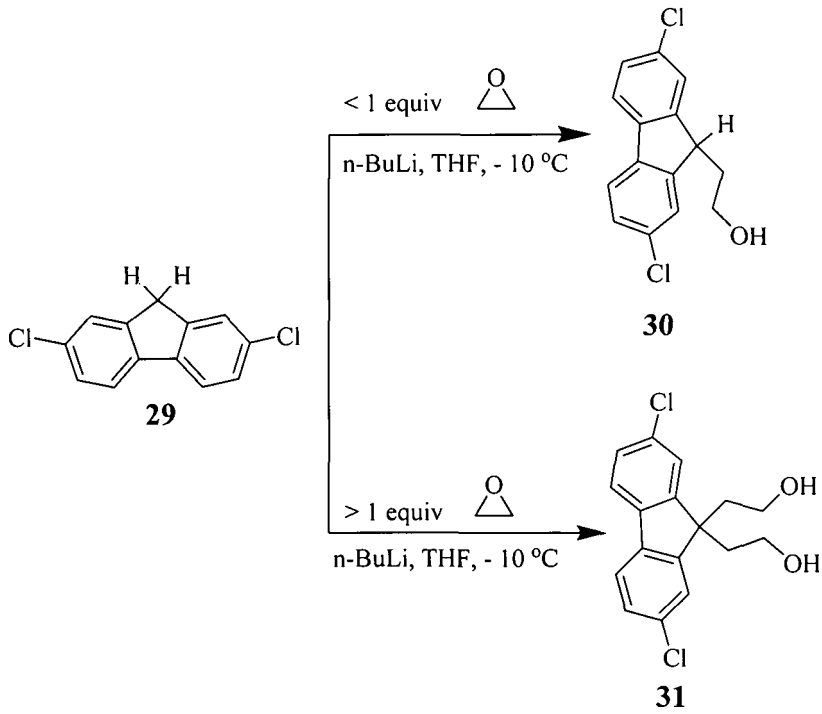
26 **27**

Fulvene formation becomes increasingly difficult with annulation, a factor that is apparent when evaluating the ease of condensation of Cp with aldehydes and ketones, formed *via* a myriad of bases including; alcoholic alkaline hydroxides or alkoxides, aqueous or alcoholic ammonia, primary or secondary amines, ethylmagnesium bromide and alkyl or aryllithiums. Indene **28** will react with ketones but requires harsh conditions. Fluorene will also react with ketones though only in the form of their bromomagnesium or lithium derivatives and not under the influence of alkoxides.[591]



28

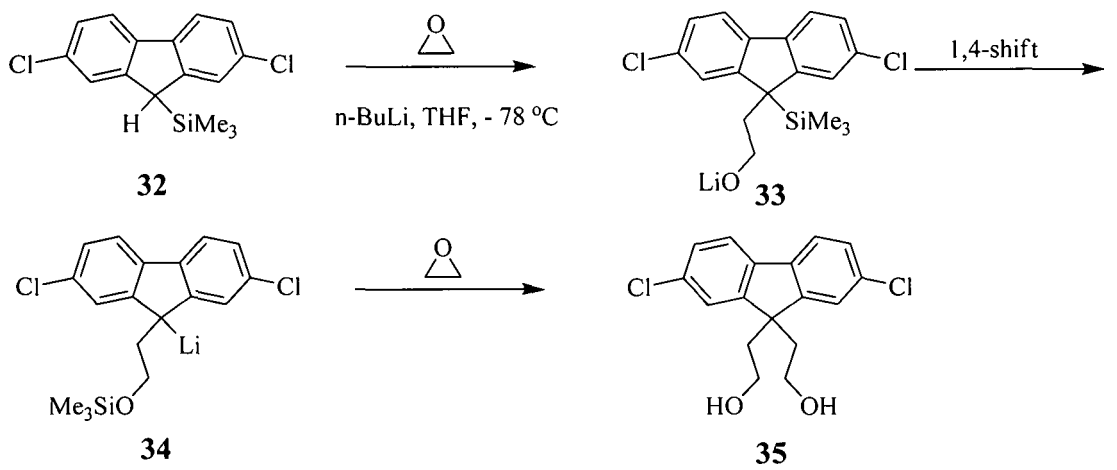
Alkylation using the 9-lithium fluorene derivative *via* treatment of a slight excess of 2,7-dichlorofluorene with butyl lithium, has revealed an alternative synthetic route to Fmoc derivatives.[570] Once the anion is generated, addition of less than 1 equivalent of ethylene oxide gives the desired product as illustrated in Scheme 19. The reaction is however sensitive to stoichiometry. If ethylene oxide is not used as the limiting reagent at –10°C, the *bis*-alkylated derivative **31** is formed as the major product since the alkoxide anion intermediate formed in the reaction can abstract another fluorenyl proton. It is likely that this proton is rendered more reactive by the addition of the alkyl group since the anion generated can be better stabilised.



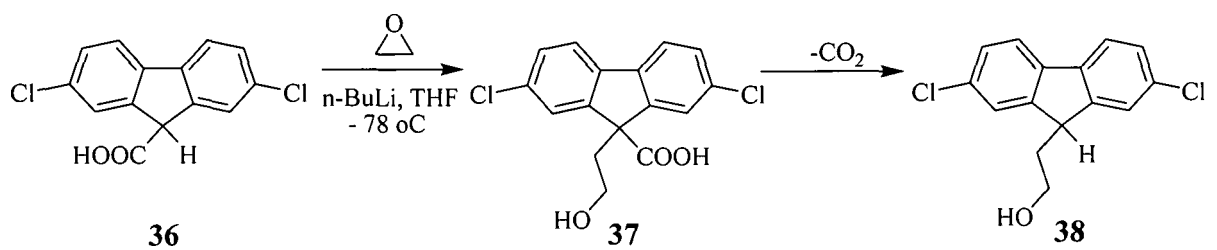
Scheme 19. Alkylation of 2,7-dichlorofluorene with ethylene oxide.

2.15.1 Metallation of Dichloro Adducts

Mono alkylation of fluorene is relatively simple. 2,7-Dichloro fluorene derivatives are however more stubborn since the *bis*-alkylated adduct **35** is more often formed as the major product due to the increased reactivity of the remaining hydrogen. Protection is viable as a way of overcoming this problem and was tried by Perumattam using TMS and carboxylation (Scheme 20 and 21).[570] However use of TMS furnished the *bis*-alkylated product due to a 1,4-shift exchanging lithium for hydrogen thereby enabling the second phase of alkylation. The carboxylic acid derivative **36** forms an unstable intermediate **37** enabling decarboxylation to give the desired compound **38**.



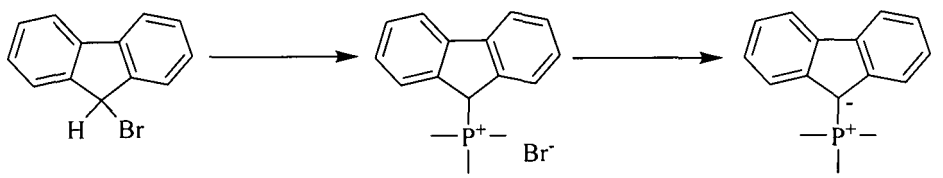
Scheme 20. Alkylation of 2,7-dichlorofluorene



Scheme 21. Formation of (9-fluorenyl)methanol from 2,7-dichloro-9-*H*-fluorene-9-carboxylic acid

2.16 Wittig Reaction

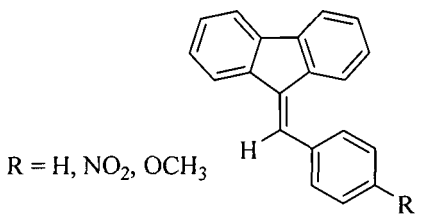
Generation of the ylide can be achieved through reaction of fluorene 9-alcohol with triphenylphosphine hydrogen bromide[624] or, *via* bromination at the 9 position to generate 9-bromofluorene (**39**) which upon treatment with trimethylphosphine furnishes trimethylfluorenyl-phosphonium bromide (**40**). Addition of phenyllithium and subsequent heating under reflux for 6hrs generates the trimethylphosphonium ylide shown in Scheme 22.[625]



39 **40** **41**

Scheme 22. The formation of a trimethylphosphoniumylide from 9-bromofluorene.

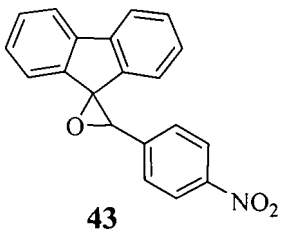
The ylide reacts with a range of carbonyl containing compounds under standard Wittig conditions. Johnson and LaCount performed the reaction to give benzylidene fluorenes (**42**).



42

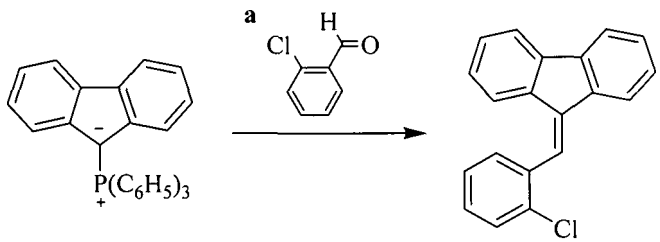
Other ylides that can be formed with fluorene include dimethylsulfonium, triphenylarsenide, tributyl (reacts with a range of carbonyls and is more reactive than triphenylphosphonium) and triphenylphosphonium (reacts best with *p*-substituted benzaldehydes).[626] The outcome of the reaction depends heavily on the choice of ylide as illustrated by triphenylphosphoniumfluoren-ylide whose selectivity is dependent on the choice of benzaldehyde and dimethylsulfonium-

fluorenylide which when condensed with *p*-nitrobenzaldehyde forms fluorene oxide **43** as the major product.[625]



43

Fletcher *et al* prepared a number of ylidene fluorenes with extended conjugation from the 9 position.[627] Initially they used sodium ethoxide for the condensation reaction between fluorene and *o*-chlorobenzaldehyde. However, the crude mixture was very hard to purify due to the presence of a side product later identified as *o*-chloro-benzylfluorene. They then looked for an alternative route and found that the formation of 9-*o*-chlorobenylidenefluorene (**45**) could be achieved in high purity by the reaction of *o*-chlorobenzaldehyde (a) and 9-triphenylphosphine fluorenylide (**44**).



44 **45**

Scheme 23. Formation of 9-*o*-chlorobenylidenefluorene from condensation of 9-triphenylphosphine fluorenylide and *o*-chlorobenzaldehyde.

A modified Wittig reaction was used by Ulmschneider *et al.* during the synthesis of inhibitors of aldosterone synthase. Potassium carbonate and 18-crown-6 were used for the coupling of fluorenyl triphenylphosphine bromide salts and various heterocycles.[624]

2.17 Reactions at the Phenyl Rings

2.17.1 Introduction

The 2 and 7 positions of fluorene are greatly activated towards electrophilic substitution due to the inductive effect and co-planarity the methylene bridge imparts.[547,548,573,628] Electrophilic substitution reactions of fluorene give predominantly 2 substituted and 2,7-disubstituted products. Direction into the 3-position can be realised by reaction at carbon-2. 2-Acetamido fluorene was successfully chlorinated to give 3,7-dichlorofluorene.[573] Nitration of fluorene in acetic acid with an excess of fuming nitric acid gives 2, 5 and 2, 7-dinitrofluorene with a small amount of the 3-isomer,[629] suggesting that the 2- and 4-positions have comparable reactivities. However to ensure substitution at the 4-position 2,7-disubstitution can be used.[582] Substitution at the 1-and 8-positions is electronically disfavoured but can be achieved by protection of the 3- and 6-positions. *Tert*-butyl groups have been employed in combination with mercury (II) as a sterically demanding electrophile for the generation of 1,8-diiodofluorenes for ligand synthesis.[630]

2.17.2 Bromination

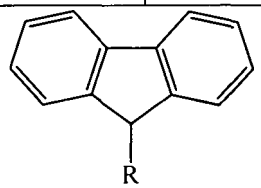
As formerly referred to, blockade of the 2 position can enable versatility on the site of electrophilic substitution. 2-Aminofluorene has been shown to undergo bromination in the 1-, 3-, and 7-positions, the position of bromination dependent on whether chloroform or pyridine is used as a solvent.[631] Bromination of fluorene is more facile than chlorination which corresponds to the yield of product obtained with NCS and NBS at 25 and 55 % respectively. At the 2- and 7-positions of (9-fluorenyl)methanol bromination has been achieved using NBS in acetic acid and HBr in an attempt to generate Fmoc analogues for use with highly base sensitive molecules.[604] Bromine, ferric chloride (99 % yield) and copper bromide on alumina in CCl_4 (98 %) have also been used.[632] Bromination of fluorene enables access to a range of products and are useful intermediates in the field of polymer synthesis.[506,533-535,572] Common methods include coupling of the bromo adduct directly with aryl zinc chlorides under palladium catalysis[535] or conversion into organometallic derivatives which are then coupled with aryl dihalides.[533]

2.17.3 Chlorination

Chlorination of fluorene can be achieved through dropwise addition of 3 equivalents of chlorine in acetic acid to furnish 2,7-dichlorofluorene as the main product offset by a small quantity of 2,3,7-trichlorofluorene. Selectivity of the reaction can be enhanced by bubbling chlorine gas through the reaction mixture in the presence of anhydrous ferric chloride.[573] NCS in acetic acid with concentrated HCl can also affect this reaction; however selectivity is compromised generating only 25 % of the 2,7 derivative.[604] More recently, chlorination of fluorene has been achieved in good yields using NCS and conc. HCl in MeCN at room temperature. This method tolerates a range of functionalities at the 9-position as represented in Table 1.[570]

Table 1. Tolerance of functionalities at the 9-position for the chlorination of fluorene

R	Yield (%)
H	90
Et	55
Br	73
Br	58
$SiMe_3$	65
CH_2CO_2H	77
$(CH_2)_2CO_2H$	52
CH_2CH_2OH	65

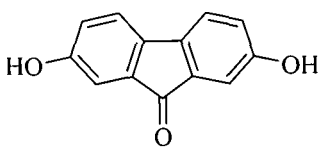


Fluorene iodination methodology is limited. A popular route to iodo fluorene utilizes fluorenone. In spite of the carbonyl group, fluorenone can be easily *mono*- and *bis*-iodinated (tri-iodination gives a product that is difficult to purify) with NIS in sulphuric acid to give 2-iodo and 2,7-diiodofluoreneone.[633] Reduction of which can give the fluorene adduct if required.

2.17.4 Nitration, Sulfonation

Nitration of fluorene does not appear to be a recurrent and exploited reaction. Again, protection of the 2-position enables a level of control over the position of substitution. 2-Acetamino-fluorene can undergo *mono*-nitration at positions 3 and 7 however the selectivity of the reaction is poor, forming the products in a ratio of 2.5:1 respectively. Control can be attained however by substitution at the 4-position *via* use of an acetamidodiphenyl group to give the 3 nitrated product exclusively.[631]

Sulfonation of fluorene was used in Andrews' preparation of tilorone and related *bis*-alkamine ethers from 2,7-dihydroxy-9*H*-fluoren-9-one (**46**). Fluorene was sulfonated using hot concentrated sulphuric acid at 100°C for 5 minutes. After which the product was neutralised as a dipotassium salt and oxidised to the oxo derivative **47**.[579]



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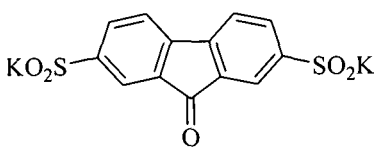


47

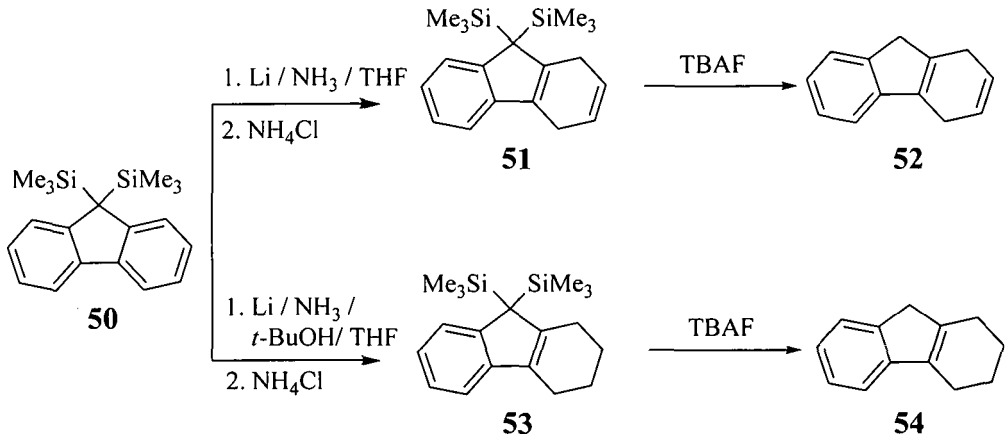
2.17.5 Reduction

Aromatic rings can be reduced using the Birch reduction reaction,[634,635] accomplished by a metal-ammonia solution to form 1,4-cyclohexadiene from benzene. Appropriate metals include sodium, lithium and potassium in alcoholic solvents such as ethanol or *tert*-butanol although the use of THF is becoming increasingly popular.[636,637] Donohoe reduced a series of hetero- and carbocyclic aromatic compounds under ammonia free conditions using lithium *bis*-tert-butylbiphenyl (LiDBB) as the electron source and *bis*-methoxyethylamine (BMEA) as the protonating agent.[636] Birch reduction is not limited to benzene and can be employed for the reduction of aromatic and poly nuclear aromatic compounds alike. The reduction of fluorene produces a mixture of isomers **48** and **49** as indicated in Figure 7 in 39 and 37 % respectively, together with 8 % unidentified material and 11 % starting material.[637]

48 **49**

Figure 7. Metal-ammonia Birch reduction products

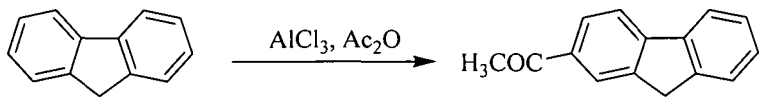
These products were difficult to isolate and purify therefore Smith applied Rabideau's findings[638] to perform the reduction using trimethylsilated fluorene in order to alter the regiochemistry of the reduced product, successfully producing one isomer dependent on the solvent and quantity of lithium used as depicted in Scheme 24.[637] The silyl groups are removed using TBAF in THF for 30 min. The regiochemical control observed could be due to the steric influence of the large TMS groups leading to the formation of one isomer **52** or **54** depending on the conditions used.



Scheme 24. Birch reduction of trimethylsilated fluorene[637]

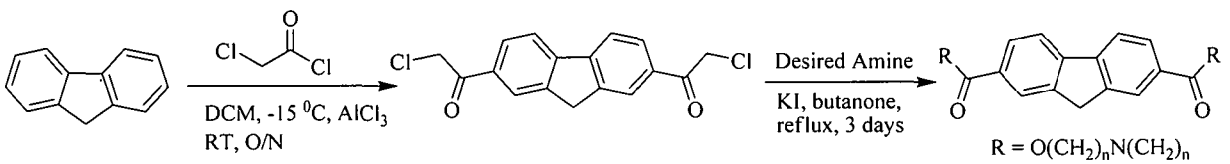
2.17.6 Acetylation

Acetylation of fluorene occurs readily under standard conditions with aluminium chloride and acetic anhydride to give the C2 acetylated product in 83%.[639] The reaction is heavily dependent on reagents and condition as illustrated by using acetyl chloride plus aluminium chloride in carbon disulfide giving compound **56** in 8% yield.[640]



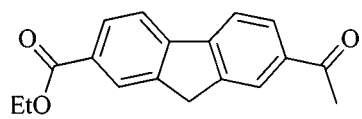
55 **56**

Friedel-Crafts diacylation of fluorene with chloroalanoyl chlorides was used by Albrecht *et al* for the formation of *bis*-basic-substituted polycyclic aromatic compounds of fluorene.[575] They used fluorene and the appropriate chloroalkanoyl chlorides to form *bis*-chloroalkanoyl fluorenes which could be aminated in the presence of excess amine to a number of aminoacyl analogues for antiviral testing (Scheme 25).



Scheme 25. The formation of 2,7-*bis*-(chloroacetyl)fluorene. This was then converted to a range of aminoacyl derivatives.

Jones also used a Friedel-Crafts reaction to affect the *bis*-electrophilic substitution of fluorene in the 2- and 7-positions as part of his synthesis of the key intermediates **57**, **58** and **59** for the synthesis of antiviral agents since this facilitated divergence from the parent structure for structure activity relationships.[641]



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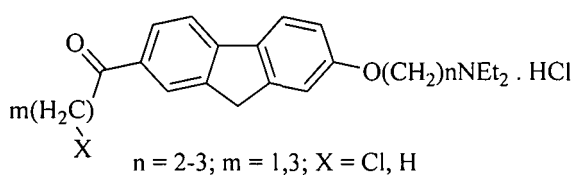

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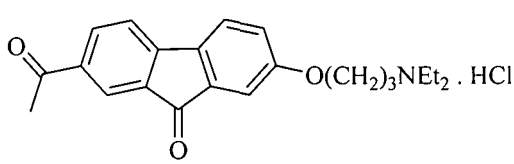
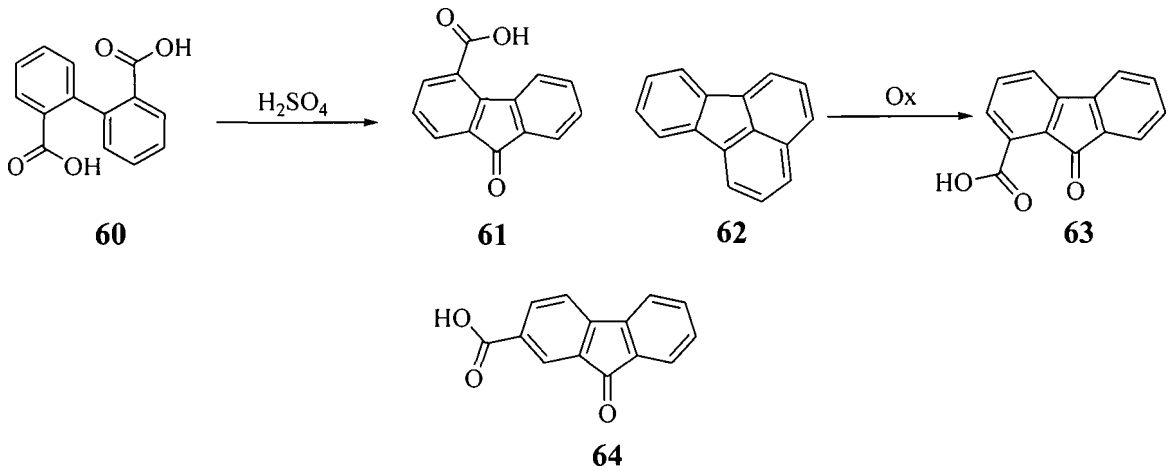


59

2.17.7 Carboxylation

Fluorene carboxylic acids can be generated by reaction at the 1-, 2- and 4-positions.[639,640] Ray and Rieveschl formed a range of fluorene carboxylic acids by heating diphenic acid **60** in sulphuric acid to form fluorenone-4-carboxylic acid **61**, oxidation of fluoranthene **62** to give the 1 substituted product **63**, and oxidation of 2-acetylfluorene to give fluorenone-2-carboxylic acid **64**.[640]



60 **61** **62** **63**

64

2.17.8 Organometallic Derivatives

Several mixed ligand metallocarboranes have encompassed the fluorene hydrocarbon[642,643] to form a sandwich complex. These complexes are synthesised through fluorene mediated thermal displacement of alky ligands generating structures of the nature in Figure 8. This validates the ease of which fluorene sandwich complexes can be generated. In addition, manipulation of the fluorene unit facilitates the generation of a diverse array of complexes.

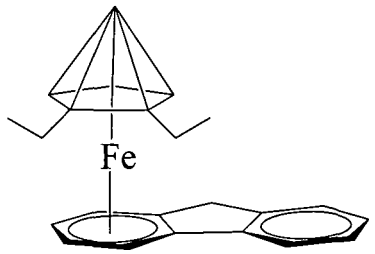
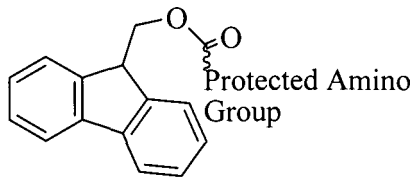


Figure 8. Metallocarborane containing fluorene

2.18 Use of Fluorenes in Synthesis (Fmoc)

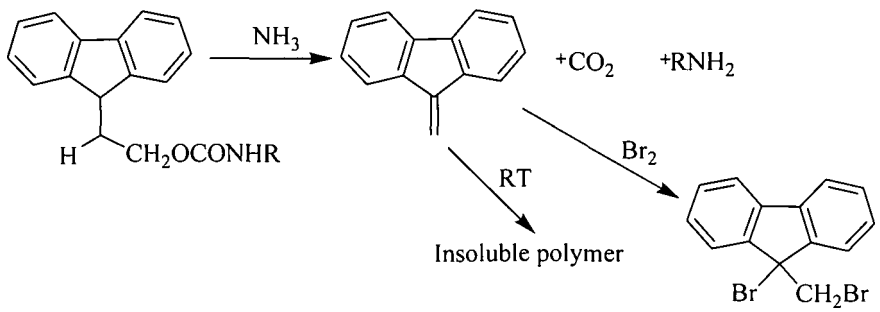
An increased interest in amino acids and their synthesis led to the development of the base-sensitive compound 9-fluorenylmethyloxycarbonyl or Fmoc **65** for use in the protection of amino groups.



65

Carpino proposed the use of the fluorenyl system during a conversation with Professor A. Ceccon[644] regarding the ease of β elimination from 9-fluorenyl thiocyanates and analogous systems relative to the corresponding benzhydryl derivatives.[607] The significance of this conversation was the development of Fmoc, a 9-fluoreneylmethyl system used for the protection of sensitive moieties. Since its development, Fmoc has been used extensively for amongst other things, the rapid assembly of peptide segments[645] and the synthesis of pentapeptides.[646] Introduction of Fmoc into the substrate is achieved by treatment of the appropriate amine with either the chloro or azido formate of 9-fluorenylmethyl with sodium carbonate in dioxane. Cleavage occurs under extremely mild conditions (Scheme 26), either by standing the reaction mixture in liquid ammonia for several hours or subsequent aqueous work-up *via* an elimination process believed to be E1cB.[608] Other convenient cleavage methods include dissolution in 4-

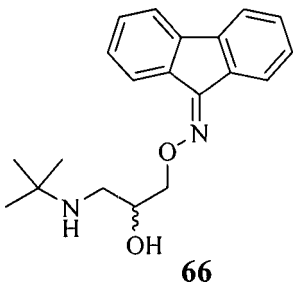
(aminomethyl)piperidine,[646] ethanolamine, morpholine or a similar amine.[607] Carpino has also modified Fmoc in order to augment accommodation of more sensitive substrates.[604]



Scheme 26. Removal of the Fmoc protecting group

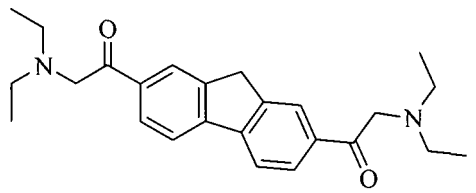
2.19 Medicinal Properties of Substituted Fluorenes

There are numerous chemotherapeutic agents that encompass the fluorene unit. Applications range from free radical scavengers[647,648] and treatment of brain edema,[649] to marketed drugs such as Lumefantrine (Lu).[650-652] Fluorenes of the type 9-R-9-aminofluorene[640] (where R is naphthyl, methyl or phenyl) and fluorenone-2-carboxylic acid, are known to possess a numbing effect when applied topically and therefore have applications within anaesthesia. Fluorenone-2-carboxylic acid boasts antispasmodic action[640,653] while the oximinofluorene **66** is a selective antagonist for the β2 adreno receptor for which there is no chiral preference since both the *R* and *S* enantiomers possess equal activity.[654,655]



66

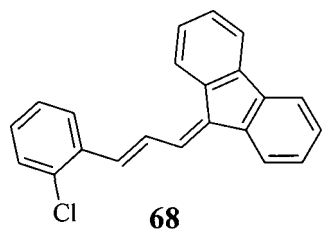
In addition to this there are many fluorene based compounds that inhibit tumour growth.[573,580,582,627,656-658] Benzyl and benzylidene fluorenes boast the ability to inhibit the aggregation of platelets,[659] *bis*-alkamine esters of fluorenone[581] and *bis*-(aminoacyl)fluorenes and fluorenones[575] induce interferon in mice and have broad spectrum antiviral properties an example of this being the orally active interferon inducer tilorone. Derivatives of fluorene have also been revealed to possess antiviral activity,[579,641] with fluorenol and fluorene based compounds being significantly less active than fluorenone based compounds.[579] However fluorene analogue **67**; 1,1'-(9H-Fluorene-2,7-diyl)*bis*[2-(diethylamino)ethanone]dihydrochloride was synthesised as an antiviral agent possessing activity both orally and parenterally.[575]



67 .2HCl. $2H_2O$

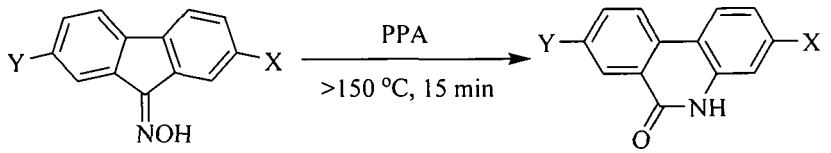
N-2-Fluorenyl mustards exhibit their greatest activity against tumour systems and low toxicity when an activating group is in the 7 position.[657]

Halogenofluorenes have been found to have antitumor properties being particularly active in the treatment of adenocarcinoma.[573,582] Furthermore, 9-*o*-chlorocinnamylidene- fluorene **68** was initially thought to have activity against animal tumour cells after showing slight antitumor activity in the mouse sarcoma S180 tumour system.[627,660] Fletcher *et al* made a range of analogues based on 9-*o*-chlorocinnamylidenefluorene but found that they did not show any antitumor activity and the activity of the title compound was never confirmed.[627]



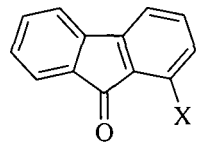
68

2,7-Disubstituted fluorenone oximes rearrange in polyphosphoric acid (PPA) as shown in Scheme 27 to produce the structurally related 3,8-disubstituted phenanthridinones. Both derivatives of fluorene were designed to enhance their efficacy by increasing the hydrophilicity of the parent halogenated fluorene, and have been shown to exhibit potent antitumor activites in a range of test systems including W256 (rat Walker carcinoma), L1210 (mouse lymphocytic leukemia cells) and S180 (mouse sarcoma).[661] The generation of *mono* halogenated phenathradinones was attempted in order to compare their activities however the oxofluorene **69** was the sole product.



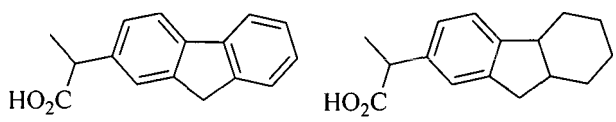
$X = NO_2$, Y = H or X = Y = F, Cl, Br, NO_2

Scheme 27. PPA mediated rearrangement of 2,7-disubstituted fluorenone oximes.



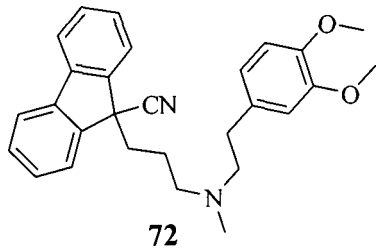
69 $X = NO_2$, I

The development of Ibufenac (1960s) and Ibuprofen (available by prescription 1969) led anti-inflammatory research in the 1970s to focus on arylacetic acids. They were the most actively investigated class of compounds in the nonsteroidal anti-inflammatory area leading to the development of non-steroidal anti-inflammatory drugs.[662-665] The arylacetic acids became a sub group in their own right, of which the commonly used anti-inflammatory agent Naproxen is a member. The anti-inflammatory activity of certain fluorenes is also documented.[662,666,667] Sprague and Heikes developed compounds based on fluorene-2-acetic acid as agents exhibiting activity in this area. They developed an analogue of their lead compound **70**, to test the effect of partial saturation of the fluorene ring system **71** on biological activity and found that anti-inflammatory activity was retained despite saturation of one of the aromatic rings with an activity comparable to Ibuprofen.[662]



70 **71**

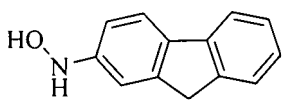
Various fluorenyl analogues were created as calcium (II) antagonists for SAR and mechanism of action studies into the effect of substitution around the quaternary carbon. Most analogues were inactive but three did show activity. Compound **72** boasted cardiovascular activity superior to Verapamil (Calan™, Isoptin™, Verelan™).[668]



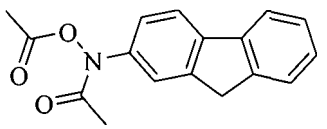
72

2.20 Toxicity

Fluorene derivatives are themselves known carcinogens particularly when containing amido and amino functional groups.[503,574,577,669,670] Namkung *et al* prepared difluoro-2-acetamidofluorene analogues for mechanistic studies regarding carcinogenicity. They established that when fluorine is in the 7-position hepatic carcinogenicity is enhanced, possibly due to blockade of a detoxification site or modification of the potency of an *N*-hydroxy metabolite which is more carcinogenic than the parent compound.[503] The metabolite *N*-hydroxy-2-acetylaminofluorene is a known carcinogen shown to bind to hepatic proteins at high levels after administration of 2-acetylaminofluorene and its derivatives.[669,671] 2-Acetyl-aminofluorene is itself a known carcinogen metabolically activated to highly electrophilic adducts that interact with DNA, RNA and proteins.[672-674] When administered, *N*-hydroxylation of acetylaminofluorene to *N*-hydroxy-2-acetylaminofluorene is the essential first step in its metabolic activation.[673] As one would anticipate the reactivity of various *N* substituted fluorenes differ. *N*-hydroxyfluoren-2-amine **73** has been found to react with guanine in nucleic acids at acidic pH while *N*-acetoxy-*N*-2-fluorenylacetamide **74** reacts with guanine at pH 7 and is involved with binding to hepatic proteins.[574]



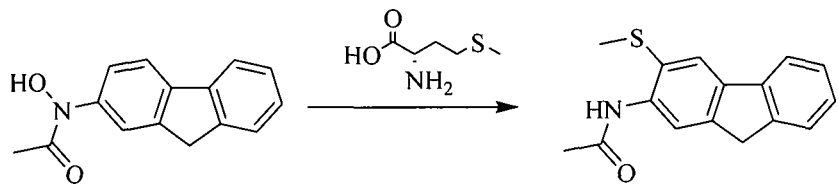
73



74

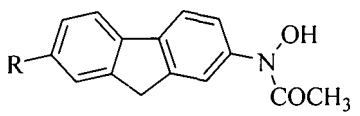
N-Hydroxy-*N*-2-fluorenylacetamide is also a known carcinogen, esters of which react *in vitro* with methionine under physiological conditions to give *N*-2-(3-methylthiofluorenyl)acetamide

which has also been isolated from liver proteins of rats administered with the parent compound.[577]



Scheme 28. *In vitro* reaction of esters of N-hydroxy-N-2-fluorenylacetamide with methionine.

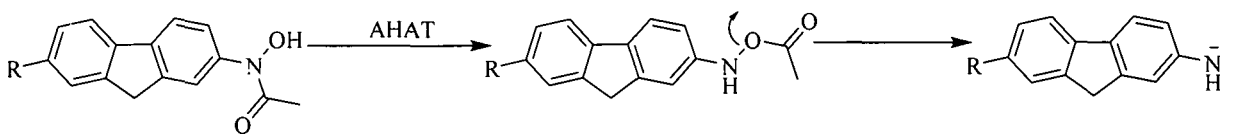
2-Aminofluorene and 2-acetamido fluorene are the most extensively used models for the study of metabolic pathways and bioactivation of fluorenes since it has long been understood that bioactivation and covalent binding to cellular macromolecules can lead to adverse effects for compounds, including teratospermia, mutagenicity and carcinogenicity.[502,504,672,675,676] Aryl amides and amines are activated to a more electrophilic species by *N*-hydroxylation. This species is then able to react with residues on cellular material. 2-(*N*-hydroxyacetamido)fluorenes (Figure 9) are bioactivated by *N*-arylhydroxamic acid *N*,*O*-acyltransferase.[675]



R = H, F, Br, I, CN, $COCH_3$, OCH_3, $O(CH_2)_3CH_3$

Figure 9. 2-(*N*-Hydroxyacetamido)fluorene. 7-Substituted analogues were generated to probe substituent effects on bioactivation[675]

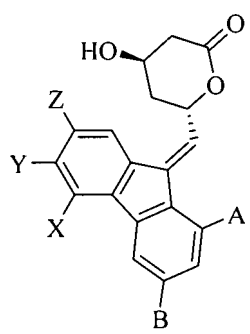
Elfarra used analogues substituted in the 7-position to probe the effect of substituents on bioactivation, concluding that electronegative substituents increased the amount of key reactive species, such as the aryl nitrenium ion, and thereby formed an increased number of adducts. This is achieved by the transfer of an acyl group from N to O by the hamster hepatic enzyme AHAT and is depicted in Scheme 29.



Scheme 29. The mechanism by which AHAT catalyses the formation of electrophilic substituents.

Inhibitors of HMG-CoA reductase have been studied in the hope of developing more efficient agents. In this search the inhibitory activities of fluorenylidene lactones **75** and biphenyl lactones **76** were analysed finding that the presence of the fluorene unit enhances reductase inhibitory activity.[559]

A = H, Cl, CH_3
B = H, Cl, CH_3
X = H, CH_3
Y = H, F
Z = H, CH_3



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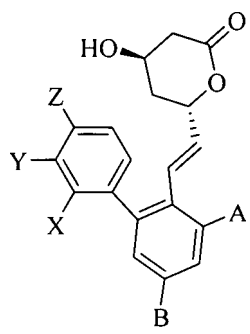
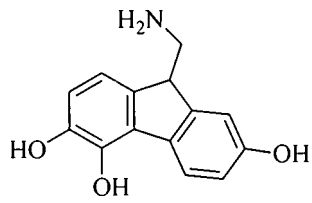


76

Aminomethyl fluorenes are also documented to be dopaminergic binders.[560] 9-(Aminomethyl)fluorenes bind weakly to D2 dopamine receptors. 2,5,6-Trihydroxy-9H-fluorene-9-methanamine **77** binds to D1 receptors in a manner comparable to apomorphine.



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CHAPTER III

PART I

Results and Discussion – Dicationic

This chapter will contain a separate numbering system for all compounds figures and schemes.

3.0 Pentamidine Analogues: Structure Activity Relationships

3.0.1 Introduction

Structure activity relationships (SAR) are frequently studied as a parameter in the development of novel drugs and have become an important primary phase in the design of novel chemotherapeutic agents. This is because they are usually based on known drugs to obtain the optimal physicochemical properties and combination of chemical moieties for biological action.

PMD is used extensively for the treatment of protozoal infections and although antimalarial activity has been observed, PMD has received minimal clinical interest for malaria. Moreover the antimalarial mode of action and accumulation of this weak membrane permeable molecule (*bis*-cationic) within the parasitised red blood cell is poorly understood.

3.0.2 Aims and Rationale

Malaria parasites use host cell haemoglobin for the acquisition of amino acids essential for parasitic growth and development. During this process monomeric heme is released and autoxidised to ferriprotoporphyrin IX (FPIX). However, FPIX is toxic to the parasite inducing lipid peroxidation[677] and destabilisation of membranes through a colloid osmotic mechanism[678] resulting in cell death.[679] However, the parasite has developed a method of detoxification by formation of malarial pigment hemozoin, formed by the crystallisation of FPIX to the non-toxic "polymer" hemozoin by hematin crystallisation. This process of heme detoxification is an established point of pharmacological intervention believed to be the site of action of the 4-aminoquinolines. For instance CQ, which by complex formation with FPIX rapidly induces lipid peroxidation more so than FPIX alone.[680] The kinetics by which this occurs is not fully understood but is believed to involve incorporation of CQ into the growing crystal thereby inhibiting crystal growth.[681]

The Bray group have previously shown that PMD is concentrated 500 fold in erythrocytes infected with *P. falciparum* and that accumulation can be blocked by inhibitors of haemoglobin digestion,[367] suggesting that the haemoglobin degradation pathway is important for PMD accumulation, implicating this process in the antiparasitic mode of action for PMD. In addition, electron microscopy studies have shown that PMD binds to hemozoin in parasitised red cells as shown in Figure 1.

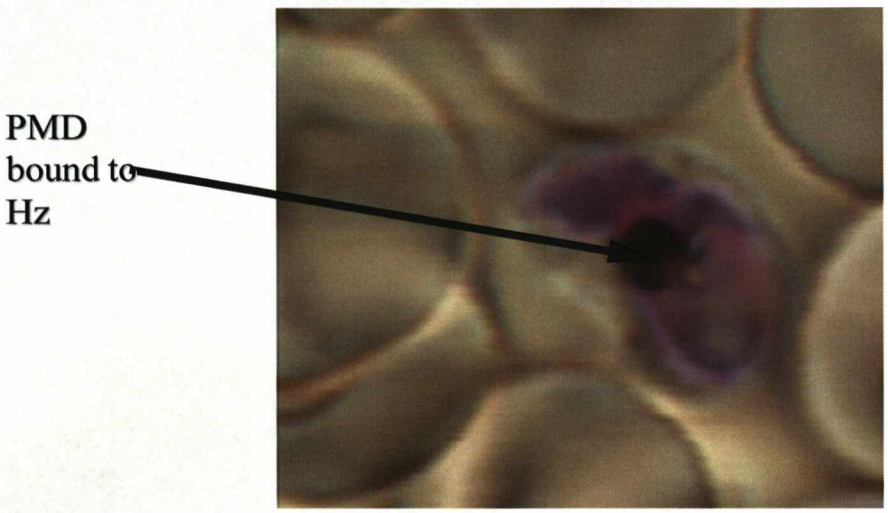


Figure 1. PMD bound to Hemozoin

PMD accumulation in *P. falciparum* has been shown to proceed through penetration of the infected erythrocyte cell membrane *via* a parasite specific process attributed to the new permeability pathway, induced upon parasitisation of the red cell leading to modifications in the cell membrane after which intracellular accumulation is achieved by transport through the choline carrier.[164] Since PMD not only exhibits antimalarial activity but also possesses a parasite specific mode of uptake, aryl diamidines demonstrate a potential new class of antimalarial chemotherapeutics and as such their development requires focused drug design. In addition, these compounds have never been synthesised by the O'Neill group before, therefore there is an additional requirement to become accustomed to their complex physical nature and chemical properties governed by their polarity.

We instigated our development of novel antimalarial aryl dications with the assessment of SAR related to PMD. Studying the PMD carbon skeleton, it is clear that there are 4 points of divergence that can be adapted for the investigation; the presence of the aryl group, linker length, alkoxy groups and *mono-* substitution *versus bis-* as shown in Figure 2.

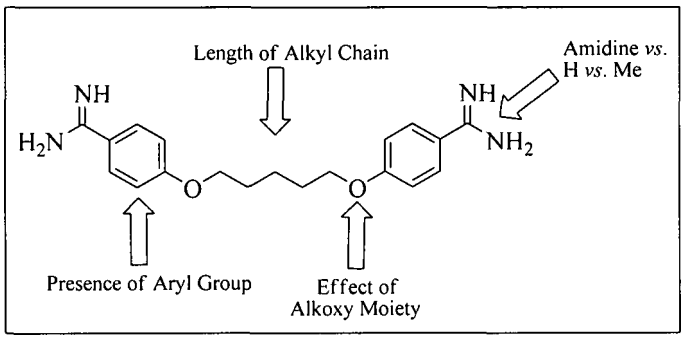


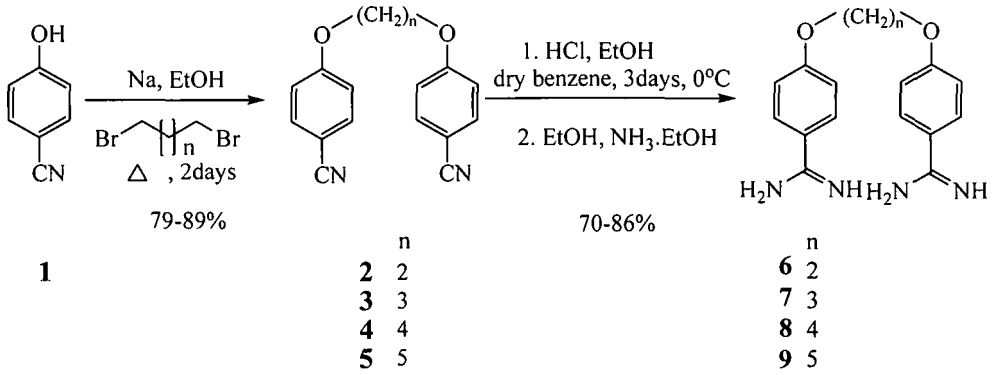
Figure 2. Points of chemical divergence of the PMD structure

Determination of the effect of these modifications on the inhibition of parasitic growth and development, in addition to their individual ability to inhibit the formation of hemozoin *in vitro* will enable focused drug design for future aryl dications. Furthermore SAR can provide increased knowledge regarding their mode of action alongside molecular modelling.

3.1 The Preparation of PMD Analogues

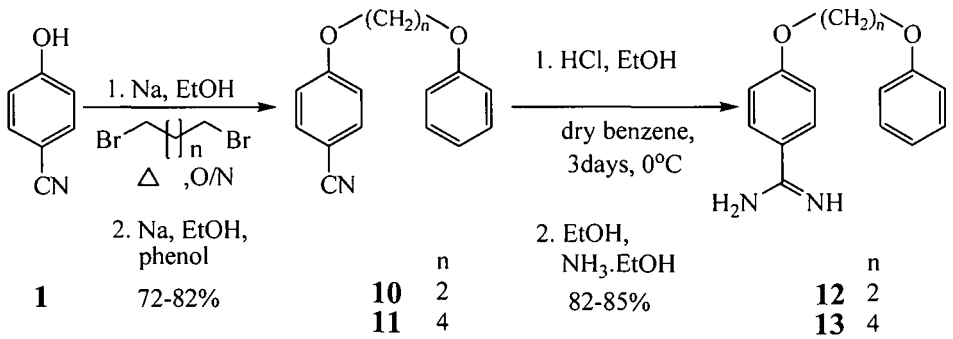
3.1.1 Alkylation

In order to determine the optimum inter-amidine separation of *bis*-benzamidines, alkyl chain length was varied using *in situ* generated sodium ethoxide as a base for the deprotonation of 4-cyanophenol as shown by Scheme 1. Subsequent nucleophilic substitution with the appropriate dibromoalkane gave *bis*-substituted nitriles **2-5** in good yields.[682,683]



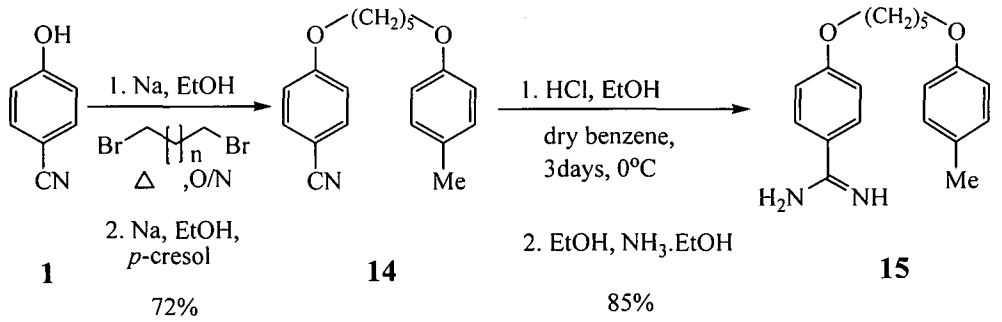
Scheme 1. Synthetic route to aromatic amidine analogues

The formation of *mono* amidines **12** and **13** is shown in Scheme 2. The reaction proceeds in much the same way as for the *bis*-nitriles, with addition of phenol after formation of the *mono*-substituted alkyl intermediate to form *mono*-nitriles **10** and **11** in 72-82 % yield.



Scheme 2. Synthetic route to *mono* amidine analogues

As depicted in Scheme 3, methyl substituted nitrile **14** is generated in a similar manner to *mono*-nitriles **10** and **11** using *p*-cresol as the secondary coupling agent to give the product in a reasonable yield (72 %).

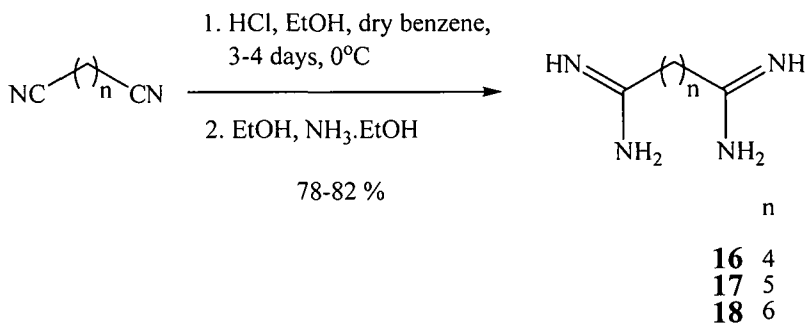


Scheme 3. Synthetic route to methyl substituted amidine analogue **15**

Initially, exclusive formation of *mono*-substituted nitriles **10**, **11** and **14** proved to be problematic, with a significant quantity of the *bis*-nitrile observed as a by-product. This problem was overcome by carefully controlled addition of phenol/*p*-cresol.

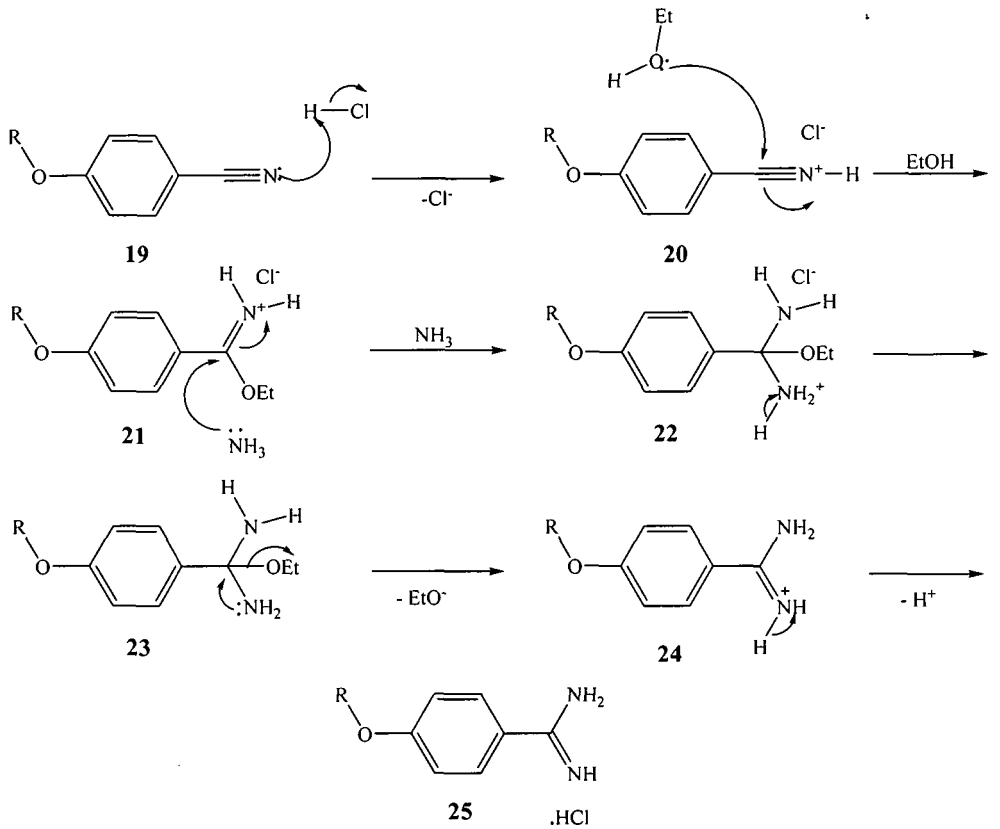
3.1.2 Pinner Reaction

Whilst looking for alternate strategies for the formation of PMD analogues, we found several examples in the literature were Pinner methodology had been employed in the preparation of amidines.[309,432,682,683] Thus Pinner methodology was used for the conversion of nitrile precursors **2-5**, **10**, **11** and **14** to their respective amidines. Alkyl amidines **16-18** were generated from their commercially available nitriles as shown in Scheme 4.



Scheme 4. Synthetic route to alkyl diamidine analogues

The Pinner reaction involves the condensation of nitrile and alcohol moieties under anhydrous conditions using acid catalysis with gaseous hydrogen chloride or hydrogen bromide.[684] The reaction mechanism as outlined in Scheme 5 involves the formation of imidate **21** generated by nucleophilic attack from the nitrogen lone pair of nitrile **19** to the acidic hydrogen giving cation **20** which itself becomes subject to attack from the oxygen lone pair of the chosen alcohol to yield imidate **21**. The imidate must be isolated under an anhydrous atmosphere after which it is reacted with ethanolic ammonia to give the cationic species **22** quenched by elimination of EtOH to give the target amidine **23**. The imidate can be reacted with an excess of alcohol to form an orthoester, with ammonia (or choice of amine) to form amidine **25** or with water to form an ester.



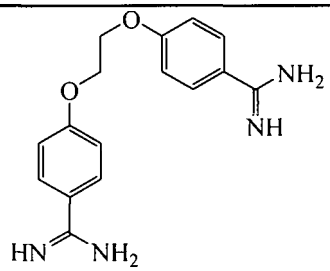
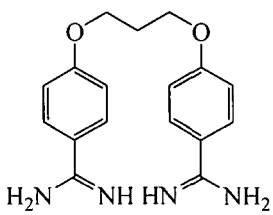
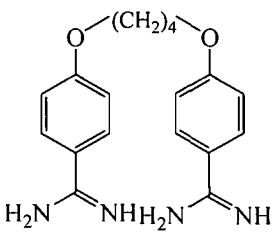
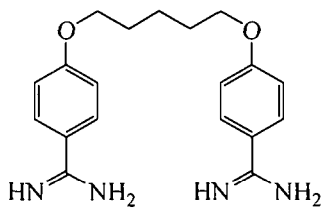
Scheme 5. Pinner reaction mechanism

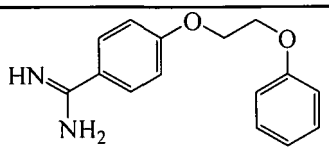
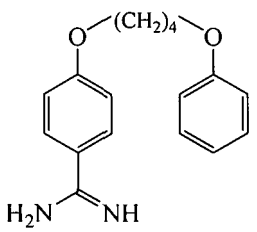
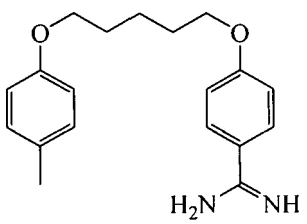
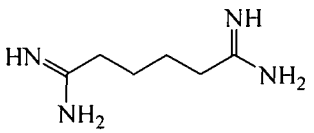
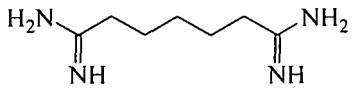
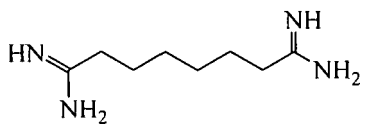
The Pinner reaction proved highly successful in the preparation of the desired amidines. One drawback to the procedure was the use of gaseous hydrogen chloride which is highly corrosive and toxic in addition to the requirement for extremely careful handling of the imidate under a

nitrogen atmosphere. Furthermore the synthetic route is linear lacking points of divergence with long reaction times; 2 days for the alkylation reaction and 3-4 days for the Pinner step.

Table 1 shows the yields of amidines **6-8, 12, 16-18** obtained from the Pinner route. Purification of Pinner reaction products **2–5, 10, 11** and **14** was most effective when formulated as the hydrochloride salt since column chromatography was unsuitable due to the polarity of these compounds.

Table 1. Synthesis of pentamidine analogues (with Lipinski's parameters)

Comp No.	Structure	Yield (%)	Formula weight	H bond acceptors	H bond donors
6		70	$C_{16}H_{18}N_4O_2$ 298.34	6	4
7		78	$C_{17}H_{20}N_4O_2$ 312.37	6	4
8		86	$C_{18}H_{22}N_4O_2$ 326.39	6	4
9		86	$C_{19}H_{24}N_4O_2$ 340.42	6	4

12		82	$C_{15}H_{16}N_2O_2$ 256.30	4	2
13		85	$C_{17}H_{20}N_2O_2$ 284.35	4	2
15		85	$C_{19}H_{24}N_2O_2$ 312.41	4	2
16		78	$C_6H_{14}N_4$ 142.20	4	4
17		82	$C_7H_{16}N_4$ 156.23	4	4
18		82	$C_8H_{18}N_4$ 170.26	4	4

3.2 PMD Analogues: Antiplasmodial Activity

3.2.1 *In-Vitro* Parasitic Sensitivity

SAR of analogues **6-18** and hexamidine (**HEX**), heptamidine (**HEPT**) and octamidine (**OCT**) were obtained in infected erythrocytes using the concentration of drug required to inhibit parasitic growth by 50 % (IC_{50}) as a measure of parasitic sensitivity reported in nM. In order to further probe the activity of these compounds their ability to inhibit hemozoin formation *in vitro* was assessed. We used *P.falciparum* from two strains of varying CQ sensitivity, TM6 (resistant) and HB3 (sensitive) as shown in Table 2 with CQ and ART as controls. As expected all analogues including PMD itself, have varying degrees of activity towards each strain of *plasmodium* being less potent than CQ and ART against CQ sensitive strains. The importance of amidine substitution i.e. *mono- versus bis-* is discussed in addition to the effect of the phenyl groups and carbon linker length. Additionally, **HEX**, **HEPT** and **OCT** (Figure 3) were previously used for similar studies by the Bray group and hence were not synthesised here, though their antiplasmodial activity profile was assessed.

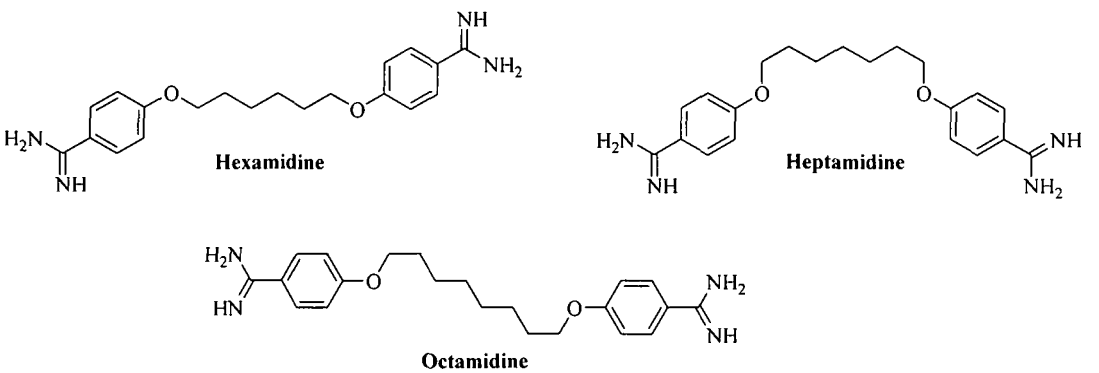


Figure 3. Structures of **HEX**, **HEPT** and **OCT**.

Table 2. Origin and CQ sensitivity of lab strains of *P.falciparum* used in this section

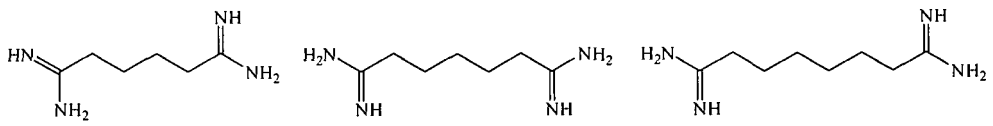
Isolate	CQ Sensitivity	Origin
TM6	Resistant	Thailand
HB3	Sensitive	Honduras
3D7	Sensitive	Unknown
DD2	Resistant	Genetically modified

Table 3. *In vitro* antimalarial activity of PMD derivatives

Compound	*IC_{50} (nM) TM6	*IC_{50} (nM) HB3
6	45	131
7	33	29
8	317	127
9	106	87
HEX	431	235
HEPT	702	334
OCT	>1000	391
12	776	625
13	>1000	>1000
15	>1000	>1000
16	>1000	>1000
17	>1000	694
18	626	815
Art	1.0	1.6
CQ	52.6	11.0

*SD within 10%; IC_{50} from an average of triplicate determinations

3.2.2 The Importance of Aryl and Oxygen Functionalities



16 **17** **18**

Figure 4. Alkyl analogues **16-18**

Three compounds **16-18** were synthesised to assess the importance of the oxygen and aromatic moieties for antiplasmodial activity of *bis*-benzamidines such as PMD. These analogues are deficient of the phenyl and oxygen moieties within PMD molecular structure, the effect of which is a marked reduction in activity against both strains of *plasmodium*. Analogue **16** was the most inactive of the alky series with IC_{50}s > 1000 nM for both isolates. Compounds **17** and **18** are however more active than analogue **16** with IC_{50}s *versus* CQ sensitive parasites of 694 nM and 815 nM respectively. Interestingly these two analogues have varying degrees of susceptibility to CQ sensitive and CQ resistant parasites with compound **18** having an activity greater than **17** to both isolates. However, compound **17** is the most active analogue of this series *versus* CQ sensitive parasites and looses activity when tested against CQ resistant parasites represented by an IC_{50} of 694 nM in the CQ sensitive test system and >1000 nM in the resistant assay. Compound **18** exhibits activity converse to this with its greatest activity against CQ resistant isolates with a reduction in activity when tested with CQ sensitive parasites (626 nM to 815 nM respectively).

The lack of activity of this series demonstrates a requirement for the phenyl group which if heme binding is involved in their mechanism of action, can be explained by the ability of planar phenyl groups to form an additional π-π stacking interaction with the planar portion of heme. An explanation for the varying susceptibility of these analogues to CQ sensitive and CQ resistant parasites cannot be clarified from this data, although clearly inter-amidine separation plays a role in the affinity of these compounds for the *P. falciparum* CQ resistant transporter (*Pf*crt) or the *P. falciparum* multidrug resistance 1 (*Pf*mdr1) polymorphism.

3.2.3 The Effect of Inter-Amidine Separation

Six compounds **6-8**, **HEX**, **HEPT** and **OCT** were assessed for the effect of the carbon linker length on parasite growth using the same system as previously described. The optimum number of carbons is shown by compound **7** as being 3 for both CQ resistant and sensitive parasites with IC_{50}s of 33 and 29 nM respectively. This is followed by compound **6** whose activity is also greater than PMD with an IC_{50} of 45 nM against CQ resistant parasites. Conversely the weakest analogue of the series is **OCT** against both isolates tested with IC_{50}s > 1000 nM for resistant parasites and 391 nM for the sensitive strain. A strict relationship between linker length and activity is not evident however, with a linker length greater than 5 carbons we observe decreasing activity as linker length increases. Furthermore this series of compounds are more active against CQ sensitive rather than CQ resistant parasites, the most prevalent example of this selectivity being OCT with IC_{50}s of 391 nM and >1000 nM respectively. Below a carbon length of 5 carbons the least active analogue is **8** (n=4), the most active being **7** (n=3) against both strains tested. In addition, compounds **6** and **7** (n = 2 and 3 respectively) were found to be more effective than CQ against the CQ resistant strain though none of the analogues including PMD were more active than CQ or ART against the CQ sensitive isolate.

Interestingly, these compounds also show susceptibility for *Pf*crt and *Pf*mdr1 mutations with the susceptibility of compound **6** to CQ resistant parasites being much greater than to CQ sensitive ones. Activity for this compound is decreased by over 3 fold when tested against CQ sensitive isolates represented by IC_{50}s of 45 nM and 131 nM *versus* resistant and sensitive parasites respectively. This specificity for parasite mutant forms actually causes compound **6** to become the least active analogue *versus* CQ sensitive isolates pre n = 5 linker length, whereas for CQ resistant forms it is the second most active compound after analogue **7**. This pattern of activity *versus* CQ sensitive and resistant isolates can also be seen with compound **8** which exhibits its most potent activity in sensitive parasites (317 nM compared to 127 nM in resistant and sensitive strains respectively). Evidently, a fixed trend between alkyl chain length and activity is not apparent pre n=5 however, inter-amidine separation clearly has an effect on anti-parasitic activity. Presumably the most active compounds are those where the chain length may allow optimum binding between the amidine and propionate groups.

3.2.4 The Effect of Amidine Substitution for Hydrogen and Methyl Functionalities

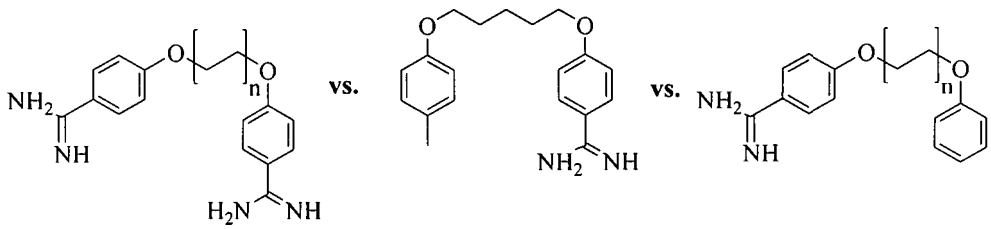


Figure 5. Analogues used to determine the effect of amidine substitution on anti-parasitic activity

Possibly the most significant structure activity effect is the exchange of one of the amidine functions for hydrogen giving a *mono*-amidine, the result of which is a significant reduction in activity. Analogues **13/8** and **12/6** are direct *mono*- and *bis*-amidine analogues of each other. Diamidine **8** has an activity of 317 nM in resistant mutants with its *mono*-amidine analogue **13** having an activity >1000 nM. This pattern is also observed for *bis*-amidine **6** with an IC_{50} of 45 nM in resistant isolates falling to 776 nM on removal of an amidine moiety (compound **12**), a trend also observed in sensitive isolates. Furthermore, for compounds **6** and **12** there is a switch in activity with compound **6** giving its most potent IC_{50} against CQ resistant parasites (45 nM *versus* CQ resistant, 131 nM *versus* CQ sensitive). In contrast, compound **12**, the *mono*-amidine analogue of compound **6**, is more potent against CQ sensitive parasites (776 nM *versus* CQ resistant parasites, 625 nM *versus* CQ sensitive). For compounds **8** and **13** a switch in activity upon *mono*-substitution is not observed since the *mono*-amidine **13** is inactive against both strains with IC_{50}s >1000 nM.

Substitution of an amidine moiety for a methyl group also results in a decrease in activity in fact, a complete loss of anti-malarial activity is observed. Compound **15**, has a methyl moiety in place of an amidine function which results in a change in activity from 106 nM to >1000 nM in resistant isolates and 87 nM to >1000 nM in sensitive ones. This series exemplifies the requirement for two cationic functions, presumably to enhance binding of these terminal groups to the terminal dianionic carboxylate residues of heme thereby locking this flexible molecule in place as shown by subsequent molecular modelling studies.

3.3 Inhibition of Hemozoin Formation

3.3.1 Methodology

In 2000, Pagola *et al.* determined the crystal structure of β-hematin using simulated annealing techniques to analyse powder x-ray diffraction data obtained from β-hematin crystals.[685] They demonstrated that β-hematin is formed by a bonding interaction between the iron and carboxylate groups in each porphyrin molecular side chain. In addition hydrogen bonding linked chains form as shown in Figure 6.

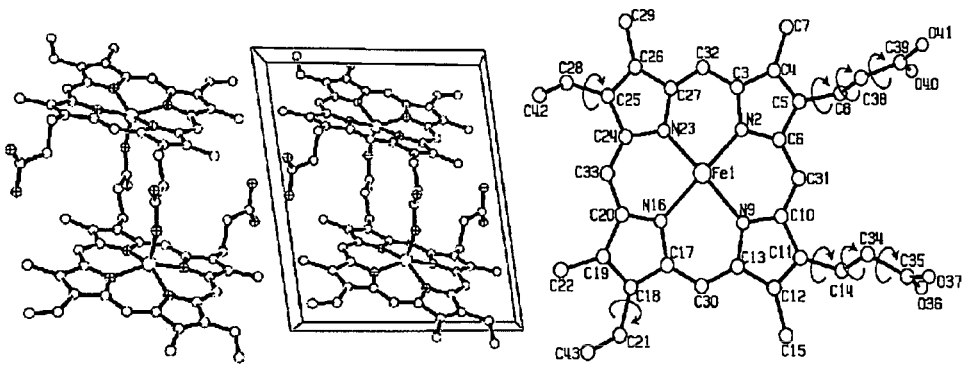
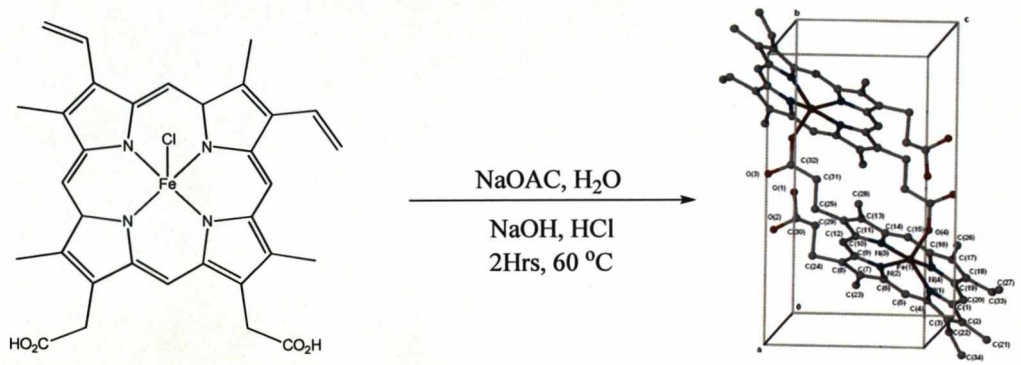


Figure 6. β-hematin and β-hematin dimer formed through iron-carboxylate bonds

Synthetic β-hematin is chemically and crystalographically identical to the malaria pigment hemozoin,[685-687] a property that was exploited during the course of our development of novel dications. By calculating the degree of inhibition of β-hematin formation *in vitro* for analogues **6-18**, we used β-hematin as a seed for the crystallisation of hemin, in a manner similar to the crystallisation of heme to hemozoin in parasitised red cells, thus allowing the determination of the effect of structural variations on the formation of β-hematin enabling focused antimalarial drug design.

3.3.2 Preparation of β-Hematin

We wanted to develop a high-throughput method using β-hematin as the crystal seed since it has been shown that β-hematin can support heme polymerisation at 37 °C.[688] Furthermore, this technique avoids the use of radioactive chemicals and laborious centrifugation steps that are used as standard test systems within our group. β-Hematin was prepared in one step from the iron containing hydrochloride of hematin, hemin as shown in Scheme 6.[689]



Hemin β-Hematin

Scheme 6. Preparation of ß-hematin

The synthesis was straightforward and gives β-hematin in a quantitative yield. Infrared spectroscopy confirmed the formation of β-hematin with bands at 1660.3 and 1209.6 cm^{-1} corresponding to the C=O and C-O stretch of the carboxylate group coordinated to the Fe (III) centre (these peaks are absent in hemin).[690,691] Furthermore, powder X-ray diffraction showed the product to be of good purity with negligible residual hemin.

3.3.3 High-Throughput Assay

The high-throughput protocol initially involved the addition of solutions of analogues **6-18** in triplicate over a concentration range to a 48-well plate,[692] although the solubility of the alkyl analogues in the suspending medium (in ammonium acetate) was poor. To this hemin dissolved in DMSO, and β-hematin as a hemozoin seed suspended in ammonium acetate, were introduced and the plates incubated at 37°C for 48 hours after which $NaHCO_3$/SDS solution was introduced

to dissolve the dimer and the absorbance calculated as a measure of β-hematin inhibition (similar to IC_{50}). We aimed to develop this system as the preferred β-hematin assay within the Bray and O'Neill groups. The method did however prove to be problematic. We repeated the above method numerous times after which we varied the reaction parameters in order to obtain an acceptable inhibition curve that was consistent and reproducible, but the results obtained were inconsistent, presumably due to the insoluble nature of β-hematin in sodium or ammonium acetate (the media required to maintain physiological pH) giving inaccurate and inconsistent β-hematin concentrations. We could no longer adapt this method within our time frame in order to move to the design of novel compounds and therefore halted the investigation into high-throughput methodology. The methods and results of these adaptations are discussed within the Appendix, Section 1. In addition, the method used to obtain hemozoin inhibition data is discussed within the experimental section.

Due to the issues encountered with the high throughput assay, we were keen to ascertain that the material synthesised was indeed β-hematin to ensure that this was not the cause of the problems. Powder X-ray diffraction was carried out by Dr J. Bacsa, University *of* Liverpool, the result of which confirms that the material used was indeed β-hematin of a high purity with cell parameters corresponding to those found by Pagola *et al.* as depicted in Figure 7.[685]

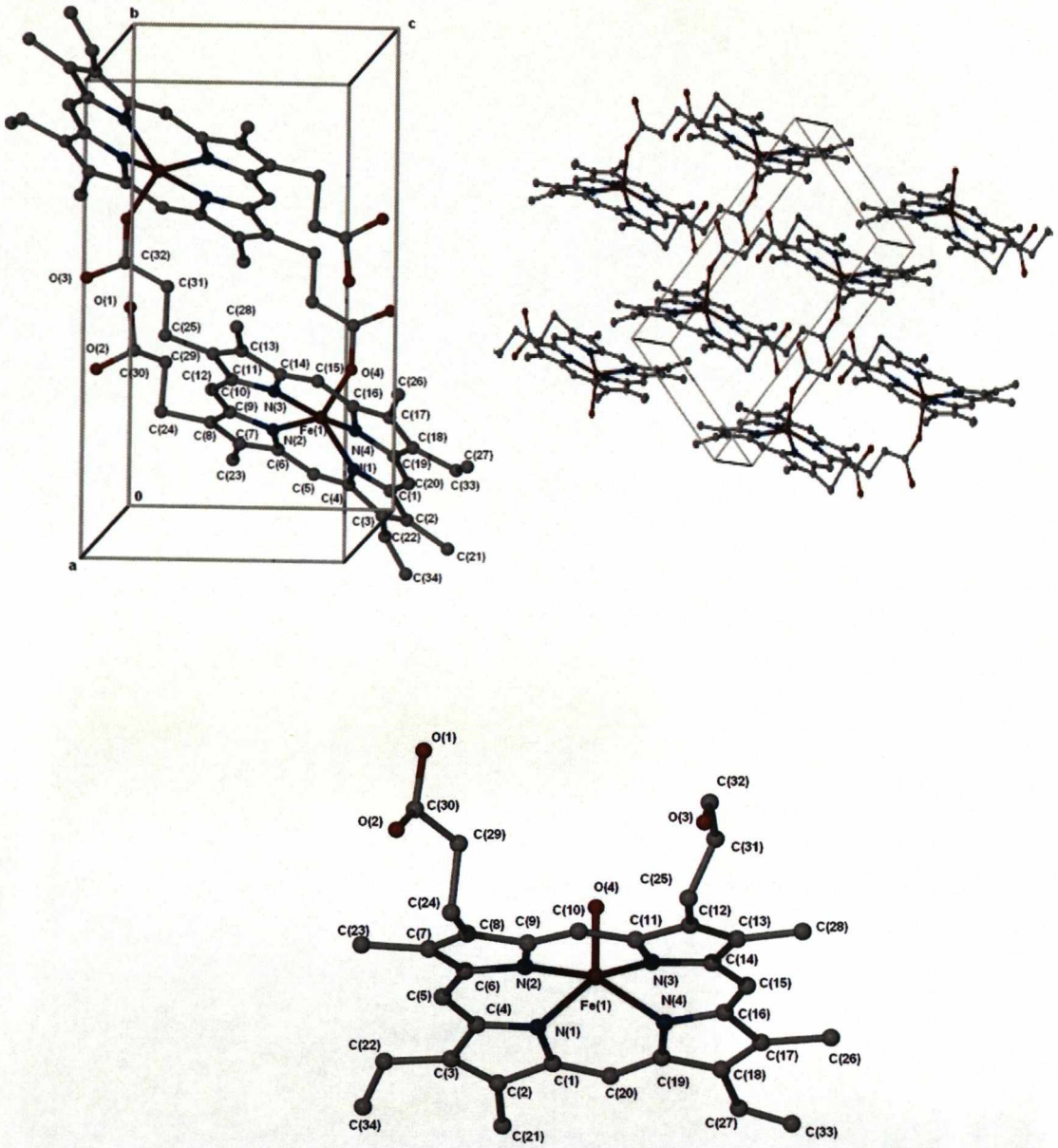


Figure 7. X-ray crystal structures of synthetic β –hematin

3.3.4 The Ability of PMD Analogues to Inhibit the *In-Vitro* Formation of Hemozoin

Table 4. Inhibition of Hemozoin formation *in vitro*

Compound	*F-P crystal growth Inhibition / μM
6	36
7	28
8	41
9	8
12	77
13	120
15	98
16	386
17	369
18	315
CQ	35

*SD within 10 % of reported figure calculated from an average of triplicate determinations

Alkyl analogues **16-18** were the weakest at preventing the *in vitro* formation of hemozoin with compound **16** being the least active corresponding to the lack of antiplasmodial activity observed for these analogues. The most active amidine aside from PMD (8 μM) is compound **7** (*bis* amidine n=3) with an IC_{50} of 28 μM, corresponding to its antimalarial activity though the antimalarial potency of this compound is greater than PMD. Compound **15** the methyl analogue, inhibits the formation of F-P crystal growth at 98 μM thereby having moderate activity within this range of analogues.

As observed for the biological activity of these agents *versus plasmodium*, hemozoin crystal growth activity decreases upon *mono*-amidine substitution as apposed to their diamidine counterparts illustrated by compounds **6** and **12** giving inhibition concentrations of 36 μM and 77 μM respectively. This pattern of activity was most markedly observed for *mono*-amidine **13** and its *bis*-analogue **8** which has a 3 fold loss in activity upon substitution of an amidine moiety for

hydrogen (120 and 41 μM respectively). These results indicate that the ability of diamidines to inhibit the formation of hemozoin is dependent on the same factors highlighted as being important for the inhibition of plasmodial growth and development, particularly *bis*-substitution and aromatic nature. This factor is shown by the most potent analogue **7** inhibiting FP crystal growth in a similar manner to CQ as depicted in Figures 8 and 9.

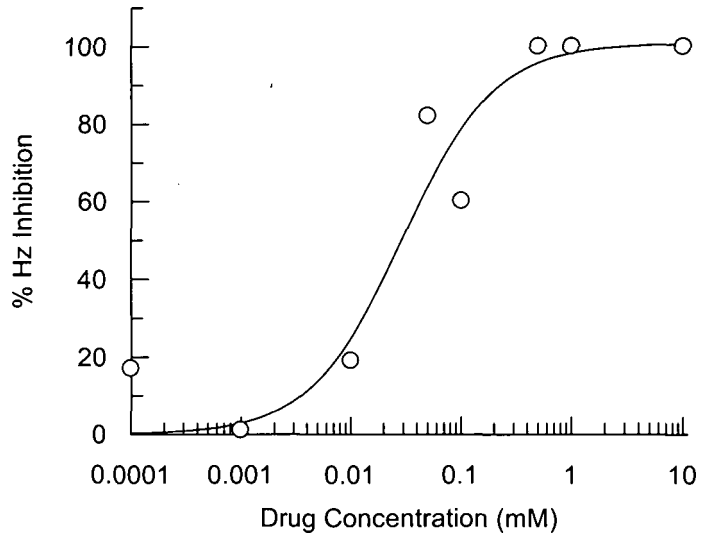


Figure 8. % Hemozoin Inhibition Curve for *Bis*-amidine **7**

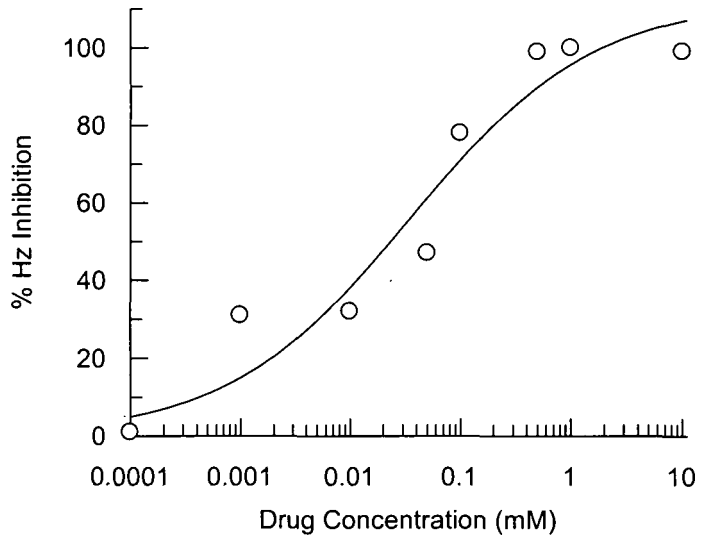


Figure 9. % Hemozoin Inhibition Curve for CQ

Clearly, a correlation between inhibition of hemozoin formation and antiplasmodial activity is evident as shown in Figure 10, inferring that inhibition of hematin biomineralisation is part of the antimalarial mode of action for *bis*-phenyl diamidines.

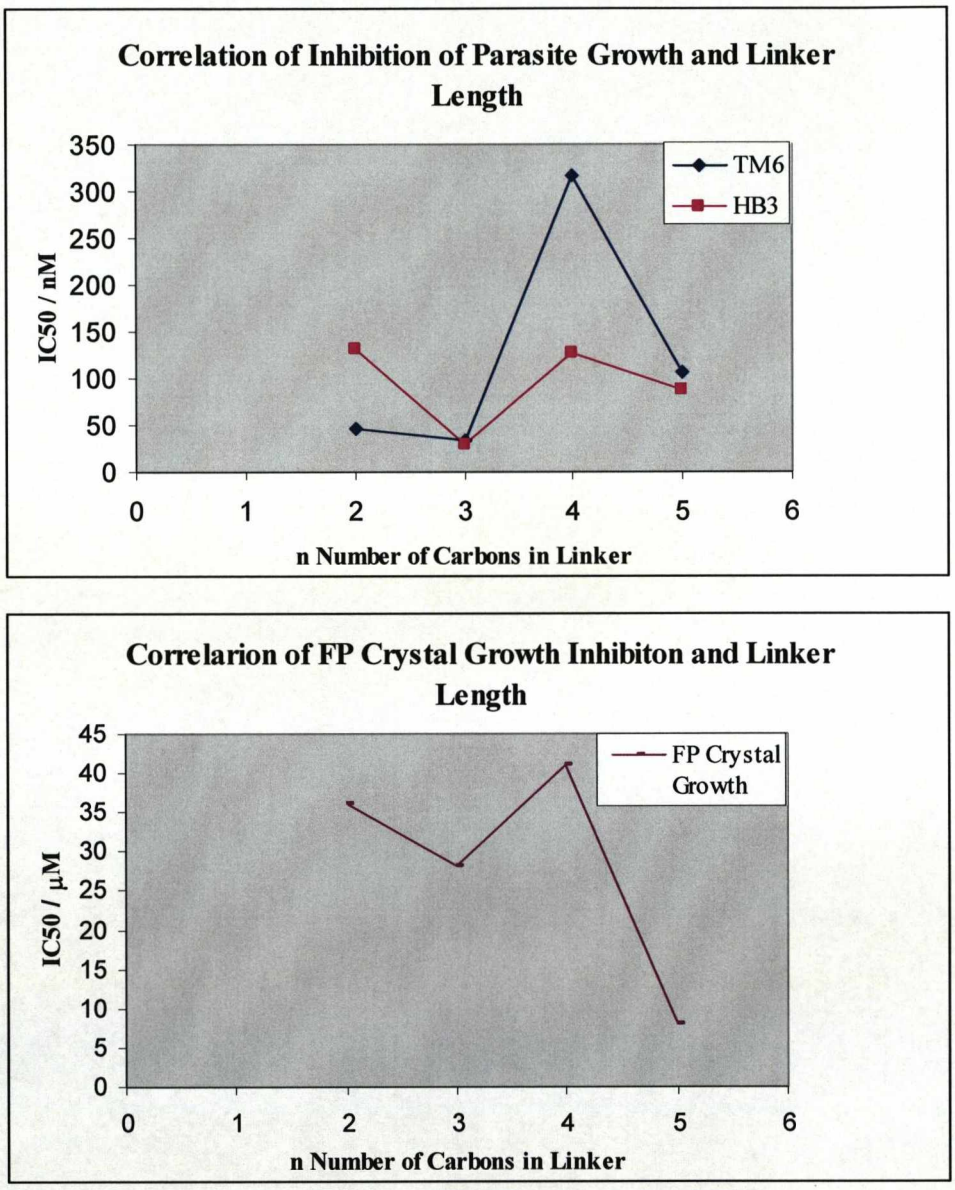


Figure 10. Relationship between inhibition of hemozoin formation and inhibition of parasite growth.

3.4 Molecular Modelling of PMD Analogues

A conformational search using a Monte-Carlo method with the MMFF94 forcefield was performed on the diprotonated molecules by Dr Neil Berry, University *of* Liverpool (for details refer to section 5.1.6). The Boltzmann weighted average of the inter amidine separation (as measured from the carbon bonded to the two nitrogens) was calculated for each molecule. These values were then plotted against $\log_{10}$(-activity) using the TM6 and HB3 data outlined in Table 3, shown graphically by Figures 11 and 12.

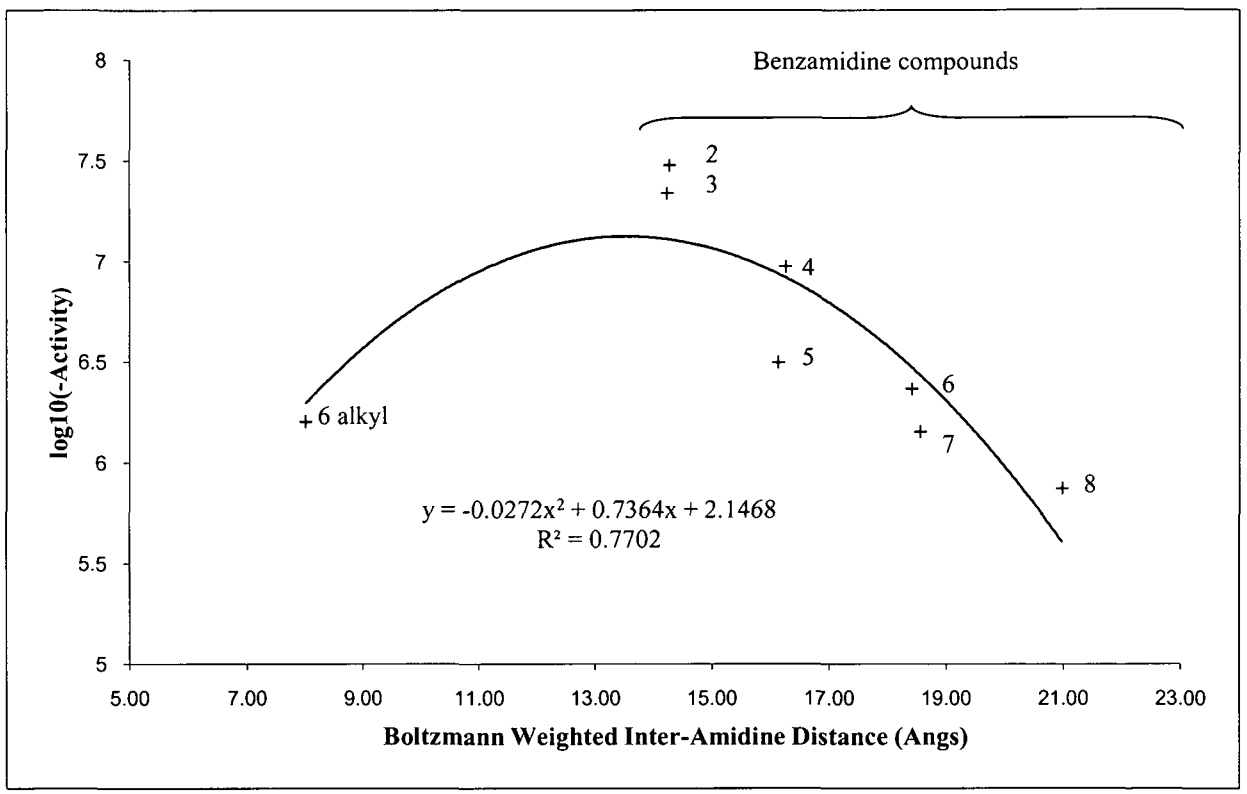


Figure 11. Plot of $\log_{10}$(-TM6 Activity) against Boltzmann weighted inter-amidine separation as a function of alkyl chain length (numbers indicate number of CH_2 groups in chain).

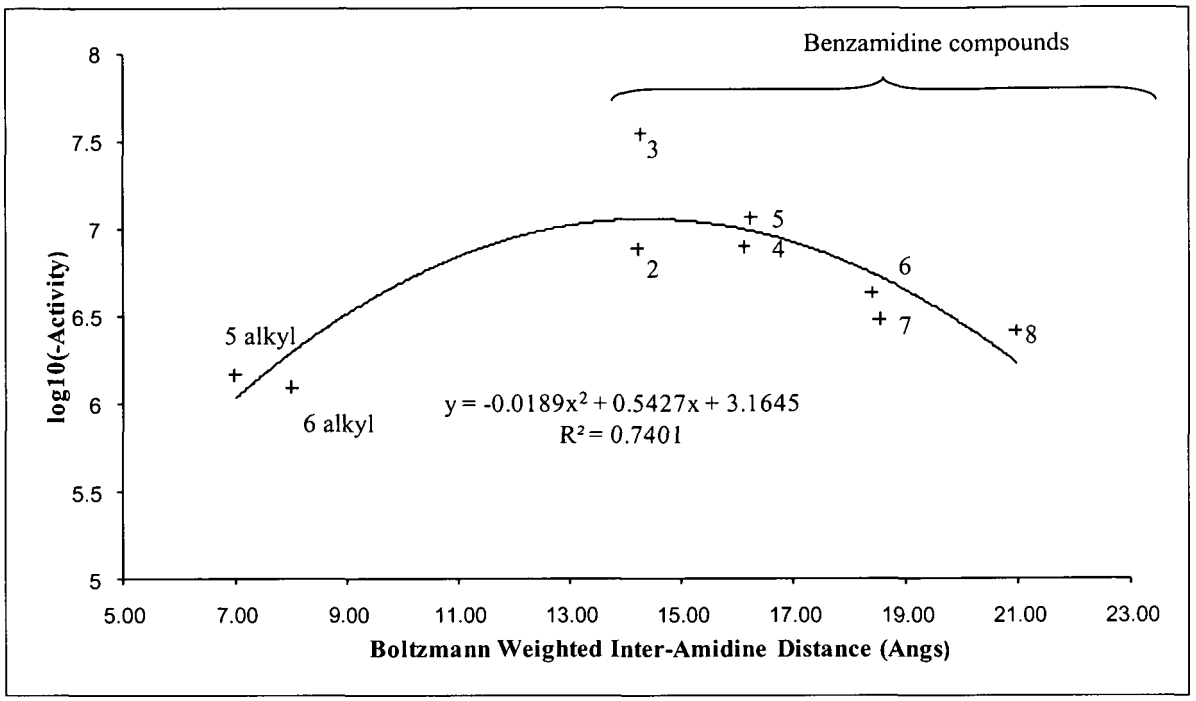


Figure 12. Plot of $\log_{10}$(-HB3 Activity) against Boltzmann weighted inter-amidine separation as a function of alkyl chain length (numbers indicate number of CH_2 groups in chain).

The results show an optimum inter-amidine separation of approximately 14 Å against both strains of *plasmodium* tested and similarities between the two strains with regards to a decline in activity for longer alkyl chains being clearly observed in Figures 8 and 9 particularly past a carbon value of n=5. Presumably a longer separation between the dicationic moieties leads to a large loss in entropy upon binding of the longer analogues to the anionic carboxylate residues of the heme template. For the *in vitro* antimalarial activity determinations we observed a loss in activity for the alkyl analogues **16**, **17** and **18**, explained by the ability of their amidine groups to bind to heme carboxylates but not its planar portion. This can also explain why *mono*-amidines have reduced activity since their aromatic rings have the ability to form a π- π stacking interaction with heme but cannot since they can bind only to one portion of the carboxylates leaving the rest of the molecule in a non-fixed orientation. There must be a requirement for both amidines in order to form a stable, ordered, heme-drug complex involving an energetically favourable interaction between the cationic amidines and anionic carboxylates of heme. Thus the binding modes of aromatic amidines and heme most likely involve a π- π stacking interaction

between the planar phenyl components and heme, a feature that corresponds to the biological activity obtained for alkyl *vs.* aromatic analogues.

PMD can adopt three different conformations, two bending and one linear, the formation of each presumably affected by which will form the favoured energy conformation. These factors were validated by a conformational search on the most active *bis*-benzamidine compound **7** (n=3) interacting with a molecule of heme as shown by Figure 13. The interaction between the protonated amidines and the carboxylates is in the form of hydrogen bonding to form a sandwich complex around monomeric heme. In addition the benzene rings do not appear to form an interaction with the entire heme template but rather a π-π interaction with the edge of heme possibly stabilising the structure. Clearly heme appears to be distorting somewhat to accommodate the binding amidines forming a complex that would have the ability to prevent the formation of reciprocal bonds for conversion of toxic heme to its non-toxic polymer hemozoin, while presumably maintaining the toxic properties of monomeric heme since its chemical constitution with respect to the central iron molecule is unchanged

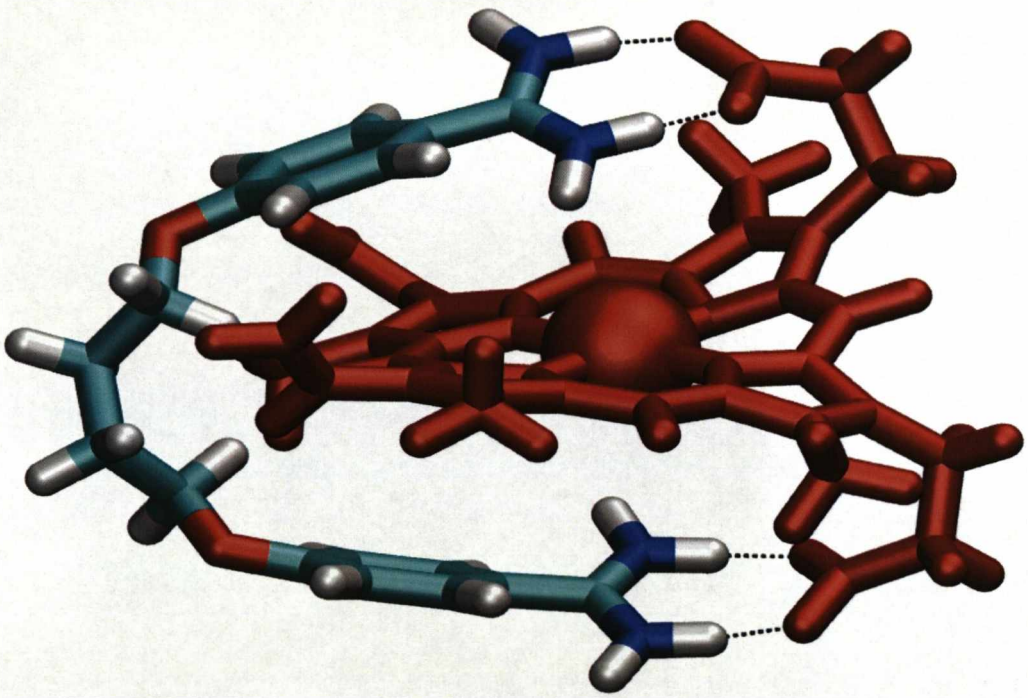


Figure 13. Lowest energy conformation of compound **7** (n=3) and heme. Hydrogen bonds are indicated with dashed black lines with the heme in red and benzamidine coloured by element; H – white, C – cyan, O – red, N – blue

3.5 PMD Analogues: Antitrypanosomal Sensitivity

The effective dose required to inhibit trypanosomal development in half the test population (ED_{50}) was calculated by Dr H. De Koning University *of* Glasgow and is reported in μM using diminazene as a control. All analogues were not equally potent having varying degrees of activity. The importance of the nature of amidine substitution in addition to the effect of the linker length and phenyl groups on WT (wild type), KO (knock out) and the resistance factor are discussed.

Table 5. *In vitro* trypanosomal activity

Compound	*ED_{50} / μM Average WT	*ED_{50} / μM Average KO	Resistance Factor[a]
6	0.55	1.39	2.54
7	0.07	0.16	2.32
8	0.02	0.04	2.17
9	0.01	0.01	1.04
12	18.13	15.85	0.87
13	2.84	1.71	0.60
15	1.34	1.27	0.95
16	IS[b]	IS[b]	IS[b]
17	IS[b]	IS[b]	IS[b]
18	60.30	92.50	1.53
diminazene	0.21	1.60	7.61

* SD within 10% calculated from a triplicate of determinations; [a]Resistance Factor – Confers degree of drug resistance, larger number = greater degree of resistance; [b]IS = Insoluble

3.5.1 The Importance of the Aryl and Oxygen Moieties

The activity of compounds **16** and **17** could not be assessed due to their insolubility in the testing media therefore compound **18** is the only alkyl analogue that was assessed. Nonetheless, the activity observed for this analogue shows a pattern similar to that observed in plasmodia, with

the absence of phenyl and oxygen functionalities resulting in a loss of activity. Analogue **18** is the least active of all compounds tested against WT mutants and KO giving ED_{50}'s of 60 μM and 92 μM respectively, furthermore the resistance factor of this analogue is slightly more than PMD (1.53 *vs.* 1.04 for PMD) but lower than some of the other analogues.

3.5.2 Further SAR

PMD (n=5) is the most potent anti-trypanosomal agent of the series followed closely by n=4 and n=3 represented by compounds **8** and **7** respectively, all of which are more active than diminazene in terms of WT and KO for the *bis*-amidines. Compound **6** (n=2) is the least active of the aromatic *bis*-amidine series with a WT ED_{50} of 0.55 μM and a KO of 1.39 μM.

The effect of *mono*- and *bis*-amidine substitution is shown by compounds **6/12** and **8/13** with a marked decrease in activity upon *mono*-substitution from 0.55 μM to 18.13 μM for **6/12** and 0.02 μM to 2.84 μM for **8/13** a trend also observed in terms of KO. Substitution of an amidine moiety of PMD for a methyl group (compound **15**) resulted in a marked decrease in activity in both test systems. The methyl substituted compound **15** was amongst the least potent antimalarial compounds, however for trypanosomes this was not the case with *mono*-amidine **12** being the least active of the benzamidine series, compound **15** being poorly active is ~18 times more potent than compound **12**. For the *mono*-amidines **12** and **13** the KO is slightly less than the WT whereas for the *bis*-amidines **6**, **7** and **8** the KO is approximately double the WT value aside from compound PMD whose values remain constant. In addition, the methyl analogue **15** also has a reduced KO when compared to its *bis*-amidine counterpart **9**.

The resistance factors obtained for the series are interesting with *mono*-amidines giving the lowest resistance factors of the series and diminazene possessing the greatest. A trend is present for RFs (resistance factor) among the diamidine analogues since as the linker length increases the RF decreases as illustrated by compounds **6**, **7**, **8** and **15**, the latter compound being the lowest. A similar trend is observed for the *mono*-amidines although substitution of H for CH_3 results in a slight increase in the RF.

3.6 Summary

We have designed and synthesised a range of (di)amidines in good yields and purity while gaining an insight into the structural requirements of aromatic (di)amidines for the inhibition of malarial parasitic growth and development, knowledge that is required for the creation of novel agents of this nature. In addition we have compared the effect of these modifications on antiplasmodial and anti-trypanosomal growth finding that the chemical functional requirements are similar for both. Furthermore, we have demonstrated the ability of these compounds to inhibit the formation of hemozoin, a possible target in the biological action of dications of this nature. Further studies to assess the requirement of the oxygen moiety directly could have been assessed, in addition to enhanced increases in alkyl chain length and variation of the oxygen moiety for other heteroatoms such as sulfur in order to obtain a more robust structure activity profile, however we felt that we gained the data necessary to move forward in our design of novel dications and therefore complete our investigation.

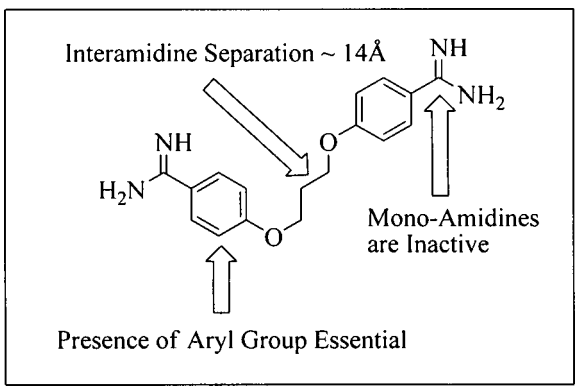


Figure 14. Structure activity profile of aromatic diamidines

The structure activity profile established is outlined in Figure 14 as requiring an aromatic moiety, in addition to *bis* amidine substitution being essential for antiplasmodial activity with an optimum inter-amidine separation of 14 Å. These analogues also display an interesting activity profile *versus* CQ sensitive and resistant parasites with some exhibiting more potent activity in the later isolates, a prospect very exciting to us as we anticipate the possibility that we may be able to access novel compounds with an ability to act preferentially on CQ resistant parasites.

3.7 Heterocyclic Diamidines

3.7.1 Introduction

Structure-activity investigations based on PMD established propamidine as a lead compound for antimalarial drug development. Specifically, aryl moieties with *bis*- rather than *mono*-amidine substitution are requirements for antimalarial activity. Furthermore, a linker length of 14Å (3 carbons) is optimal for antiplasmodial potency. It is the nature of this linkage that we wish to establish as illustrated by Figure 15.

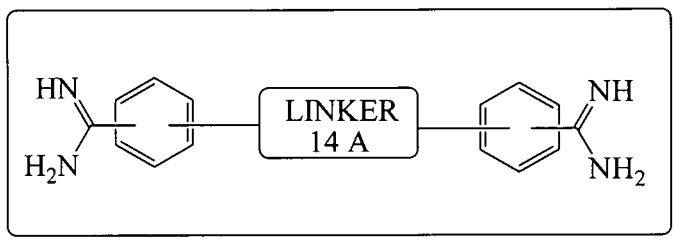


Figure 15. Diamidine Drug Template 1

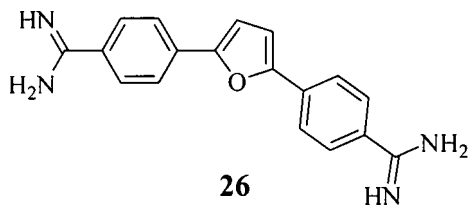
3.7.2 Rationale

Through *in silico* molecular modelling, we established that the flexible carbon linker of propamidine aids the formation of the drug-heme 'complex'. In addition to this, the amidine moiety interacts with the heme propionate groups. With this in mind, our aim was to enhance the affinity of these molecules for heme. In order to achieve this, molecular spatial arrangement is an important factor. *Bis*-benazmidines such as PMD are flexible due to the possibility of rotation around the central carbon linker. It has been revealed that analogues of PMD with a double bond present show an enhancement in anti-protozoal activity,[693,694] presumably the rigidity imparted by the double bond aids binding to the bio-molecular target.

The locking of flexible molecules is well established within chemotherapeutic drug design, as an approach to enhancing the specificity of a drug molecule for its site of action, a feature commonly used for lead optimisation. In addition to double bonds, heterocycles have also been used to form conformationally restricted diamidines.[304,305,309]

3.7.3 Proof of Concept

A furan heterocycle was employed by Das and Boykin as a linker between the two phenyl amidine groups giving DB75 (**26**), a molecule with potent anti-trypanosomal activity.[452] In addition, this compound has potent antimalarial activity *in vitro versus P. falciparum* CQ resistant isolates (15.5 nM, K1)[695] with weak toxicity to mammalian cells. Furthermore DB289, the dimethoxy prodrug of DB75 has been tested for the treatment of *P. vivax* and acute, uncomplicated *P. falciparum* infections with success.[492]



26

Figure 16. Structure of Furamidine (DB75)

As mentioned, structure activity relationships denote an inter-amidine separation of approximately 14Å (given by a carbon linker of 3) for optimum activity. A requirement that can be achieved by use of a five membered heterocycle furthermore, doubly atom containing heterocycles can act as isosteres for the two oxygen atoms linking the benzamidines of PMD as shown in Figure 17.

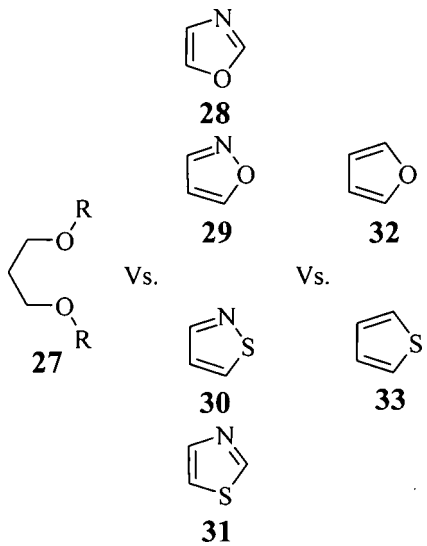
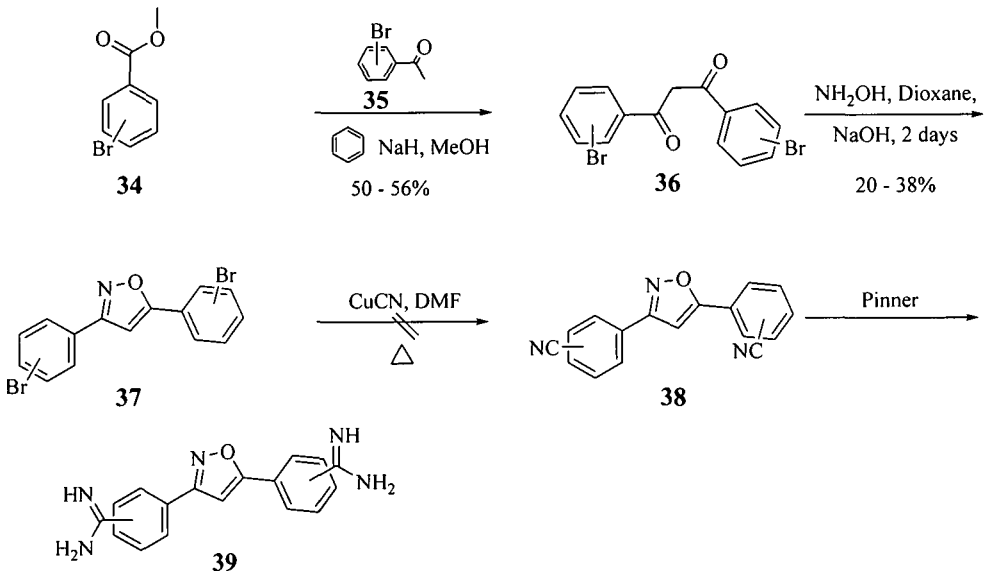


Figure 17. Lead Compound Propamidine and Isosteres

Clearly heterocycles **28-33** have similar molecular shapes and distributions of electrons, more so than **32** and **33**. In addition to locking of the central linker, variation of the amidine position on the ring may exert an effect on antimalarial activity therefore isomers will also be assessed.

3.8 3,5-Diphenylisoxazole

3.8.1 Chemistry



Scheme 7. Intended synthetic route for the formation of isoxazole diamidines

The synthetic route utilised is outlined in Scheme 7. The first step of the sequence involved the condensation of the appropriate commercially available bromobenzoate **34** and bromophenyl-ketone **35**, using sodium hydride in the presence of a small amount of methanol.[696] 1,3-Diketones **36** were formed in moderate yields. However, the generation of these compounds was not straightforward. The work-up of the reaction mixture was problematic due to the formation of an emulsion during the extraction process which rendered separation of products difficult. We found that benzene was the only suitable solvent for product extraction from the reaction media, since solvents such as Et_2O, DCM and chloroform failed to adequately separate aqueous and organic fractions thereby minimising emulsion formation. In addition to these factors, purification of the product was compounded by its insoluble nature in most organic solvents.

Purification was however achieved by multiple recrystallisations from benzene and suspension of the product in hot EtOH.

Studying the literature, common methodologies for isoxazole formation involve the attack of hydroxylamine on a molecule containing three-carbon atoms for instance a 1,3-diketone, a α,β-dibromoketone or intramolecular cyclisations of amino acids.[695,697] Diketone **36** was converted to isoxazole **37** upon treatment of a suspension of **36** in dioxane with aqueous sodium hydroxide and hydroxylamine heating to reflux over 2 days after which the product was purified by column chromatography.[698]

The conversion of dibromoisoxazoles to their dinitrile counterparts was a key step in the synthesis of target molecules. However the reaction was hampered by the generation of copper halide salts and this made product isolation difficult. We commenced our conversion of dibromoisoxazoles to their dinitriles using the method of Das and colleagues.[452] Das used one equivalent of $Cu_2(CN)_2$ in refluxing quinoline to form a *bis*-nitrile derivative. This methodology was attractive since this was used for the cyanation of a closely related aryl furan compound. However $Cu_2(CN)_2$ could not be purchased thus we replaced one equivalent of $Cu_2(CN)_2$ for two equivalents of Cu(II)CN (Table 6, entry 1). However, starting material and the *mono*-nitrile were recovered, which we assumed to be due to insufficient CuCN thus we repeated the reaction with 4 equivalents of CuCN again in quinoline however after laborious column chromatography steps the *mono* substituted product was again isolated. Following the failure to achieve the desired transformation we then considered the Rosenmund Von Braun (RVB) reaction. The RVB reaction involves cyanation of aryl halides using an excess of copper (II) cyanide in a polar high-boiling solvent, most frequently DMF since the reaction requires high temperatures (150-280°C).

Table 6 shows the reaction conditions used in an attempt to improve the formation of the *bis*-substituted product. Modifications include the number of CuCN equivalents, reaction duration and temperature. We were cautious not to elevate the temperature too much over concerns over the stability of the weak N-O bond. In addition to these parameters, the work-up procedure was varied in some cases to include pouring the cooled reaction mixture into solutions from 10% NH_4OH to conc. NH_4OH and pouring the hot reaction mixture into a solution of hot KCN (10%)

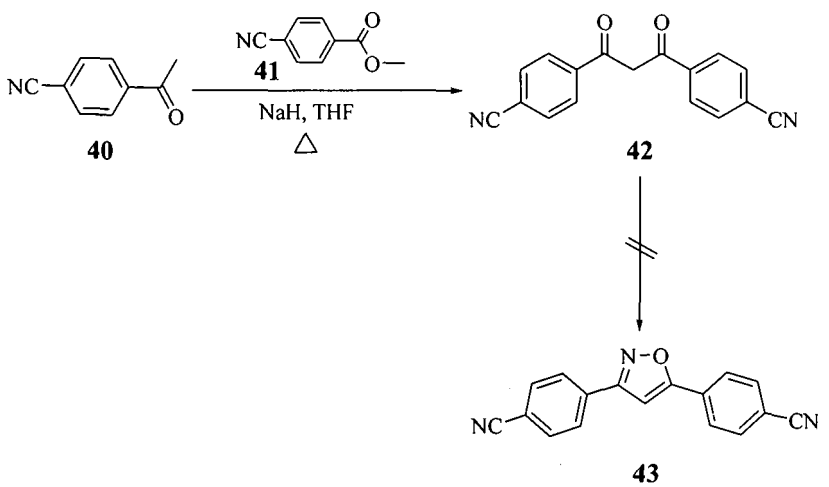
after which the mixture was stirred at room temperature overnight. The TLC showed the reaction mixture to be *mono*-nitrile and starting material. We also tried various methods of combining the mixture from sequential addition, to combination of the isoxazole and CuCN in a pestle and mortar, to pre heating in DMF all of which resulted in recovery of the starting material and *mono*-nitrile. Unfortunately, in all cases degradation occurred.

Table 6. Variations of reaction conditions used for the conversion of dibromoisoxazoles to nitrile

Entry Number	Reaction Time / hrs	CuCN / Equivalents	Temp / °C	Yield of *Mono* Product (%)
1	17	2	150	15
2	5	4	190	ND*
3	17	4	190	24
4	21	4	150	19
5	4	13	150	ND*

ND* Not Determined

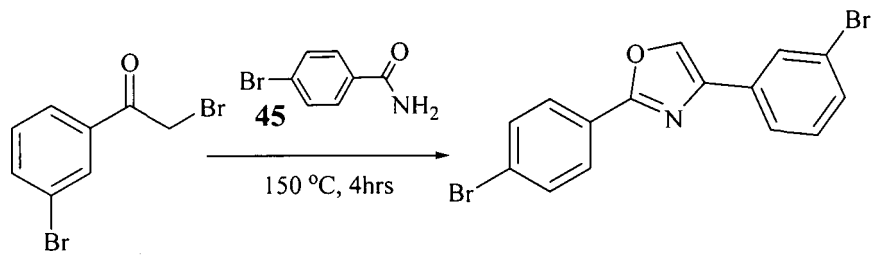
Having met with failure with the CuCN approach we decided to investigate an alternate strategy where the nitrile function is in place at the start of the synthesis.



Scheme 8. Alternative strategy to the formation of dinitriles

Claisen condensation was achieved by combining methyl 4-cyanobenzoate **41** and 4-acetylbenzonitrile **40** in THF in the presence of NaH. Although **42** was produced in good yield (76%), access to the isoxazole *via* subsequent ring closure was not accomplished by TLC. This was also confirmed by the presence of the dicarbonyl proton singlet in the ^{1}H NMR spectrum.

We considered that the oxazole structure would have enhanced stability and therefore its analogues could have superior *in vivo* activity. The synthesis of oxazole **46** had an additional advantage that access to the heterocycle was in one step. The procedure involved heating ethanone **44** and bromobenzamide **45** at 150°C for 4hrs.[699]



44 **46**

Scheme 9. Oxazole strategy

Oxazole **39** was shown by ^{1}H NMR to be the synthetic product however its isolation was so problematic that we assessed alternative heterocyclic linkers.

The thiazole unit has been employed leading to the generation of *bis*-thiazolium salts, some of which show potent antiplasmodial activity, their suggested mode of action being inhibition of choline transport.[155,156] T16 is a potent heme binding antimalarial that accumulates several hundredfold into parasitised erythrocytes.[157] In addition, the hydroxy analogue of T16, T3, has been developed as the prodrug TE3 possessing potent oral activity with an ED_{50} of 5 mg/kg as shown in Figure 18.[158]

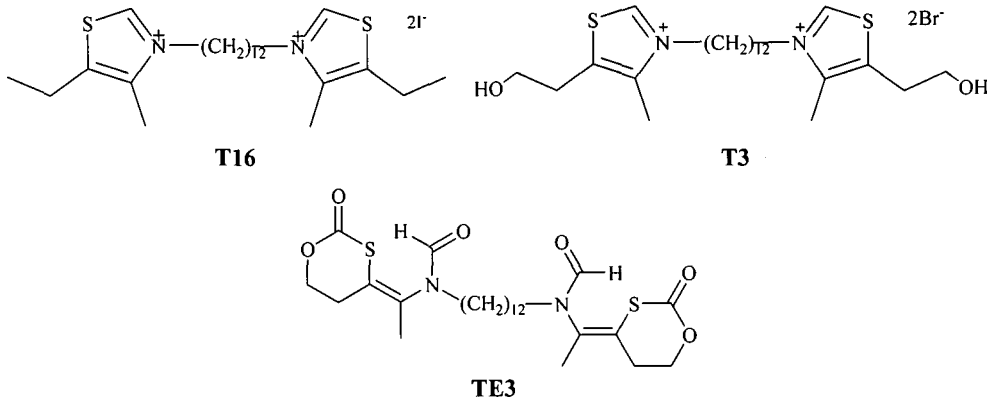
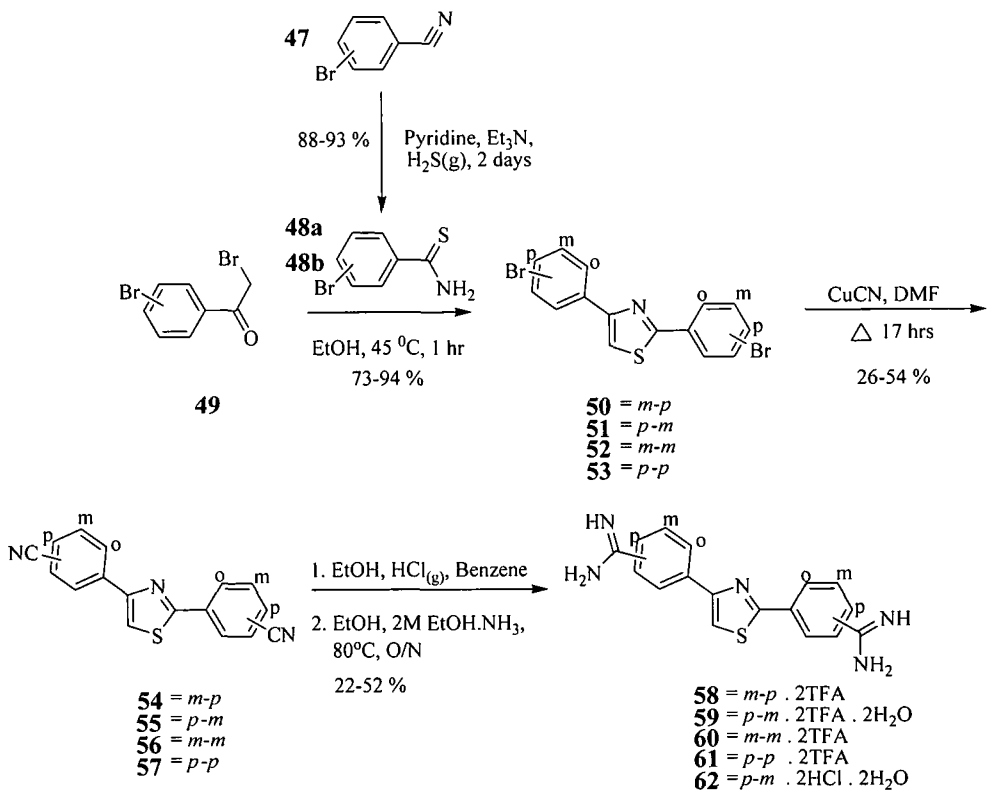


Figure 18. Structures of T16, T3 and prodrug TE3

3.9 2,4-Diphenylthiazoles

3.9.1 Chemistry

The synthetic route designed for the generation of 2,4-diphenylthiazoles is outlined in Scheme 10.

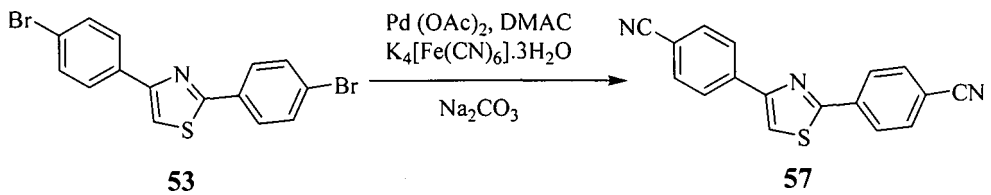


Scheme 10. Synthetic route to Thiazole analogues. TFA salts generated by reverse phase HPLC

Using the Hantzsch synthesis for the construction of the thiazole unit, thioamide **41** was condensed with α-haloketone **42**, the thioamide being prepared from the reaction of nitrile derivative **40** and gaseous hydrogen sulphide (H_2S) in triethylamine and pyridine.[700] Subsequent condensation of α-halo ketone **42** with thioamide **41** afforded the precipitation of dibromothiazoles **43-46** in excellent yield after warming to 45°C for 1 hr.[700]

Generation of *bis*-nitrile derivatives **54-57** once again proved to be difficult. The desired *bis*-cyano thiazoles were prepared after refluxing the appropriate dibromothiazole overnight in a mixture of CuCN and DMF[450] in yields ranging from 23-60% dependent on the isomer. The reaction was again subject to the sensitivity of the reactant and product under the reaction conditions, degradation products and isolation of the *mono*-nitrile were commonly observed. The *bis*-nitrile however was formed for all analogues in one cyanation step and the crude material purified by column chromatography. In our hands the success of the reaction was dependent on the reaction temperature and the removal of DMF and copper halide salts generated during the reaction process. Specifically, the temperature must be maintained at 150 °C; lower temperatures give an increased recovery of the starting material and *mono*-nitrile, at temperatures above 150 °C degradation occurs. The generation of copper halide salts during the reaction process complicated the purification of the crude material therefore their separation from the nitrile products by stringent washing and filtering processes were imperative due to the low solubility of the nitrile crude mixture for column chromatography. In addition the insolubility of the nitrile in the Pinner reaction media means that any residual inorganics cannot be removed. Furthermore the use of polar, high-boiling DMF as solvent complicates extraction/washing processes and purification of the nitrile and therefore its removal from the crude reaction mixture by evaporation to dryness overnight (high vac) prior to column chromatography was crucial for good separation.

Due to the difficulties encountered with the cyanation reaction, whilst screening the final analogues alternate methodology for the generation of the nitrile was briefly assessed. Weissman *et al.* report methodology for the ligand-free conversion of a bromo moiety to a nitrile function in a mild manner.[701] They apply palladium catalysis using palladium acetate in dimethylacetamide with potassium hexacyanoferrate as the nitrile source as shown in Scheme 11.

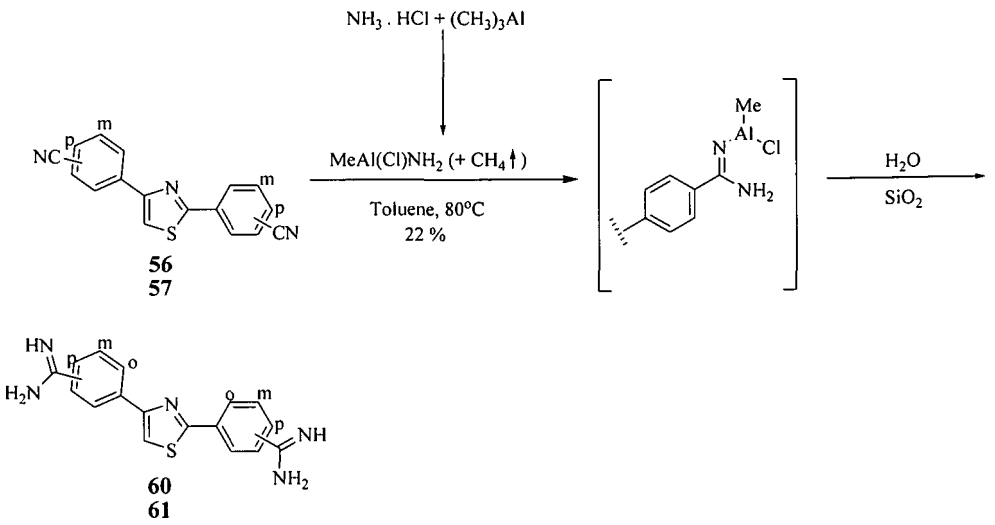


53 **57**

Scheme 11. Alternative route to the cyanation of dibromothiazoles

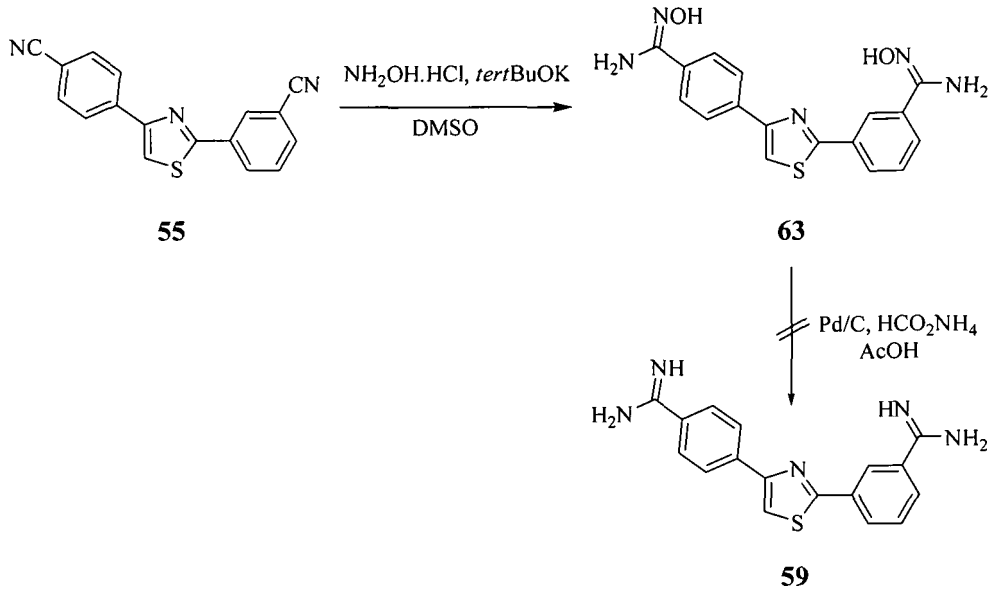
The course of the reaction was monitored by TLC, product formation was initially observed, ceasing after a few minutes; presumably the thiazole moiety was poisoning the catalyst thus rendering this methodology unsuitable. Nonetheless the reaction was applied to the formation of dicyanoisoxazoles and again monitored by TLC. After 5-10 minutes formation of the *bis-* nitrile was observed. At this point however, we did not move forward with this methodology due to our focus on the thiazole heterocycle.

Pinner methodology was again employed for the transformation of nitriles **54-57** to their appropriate diamidines **58-62** however this was troublesome due to the partial solubility of the dinitriles in the appropriate solvent media. Over the period of the reaction we also observed the formation of the *mono* substituted product precipitating of solution. As opposed to the PMD analogues previously synthesised, the conversion of thiazole nitriles to diamidines required a minimum of 1 week for the formation of the imidate. The extended reaction times, careful handling of intermediates under an inert atmosphere, low yields and difficulty with purification of the diamidine product led us to briefly assess alternative methodology. Routes to the formation of diamidines by methods other than the Pinner reaction are limited; however Garigipati found that alkylchloroaluminium amides can affect the transformation of amidines from a nitrile precursor in one high yielding step by the direct nucleophilic addition of an amine to a nitrile.[702,703] Accordingly we applied the Garigipati reaction for the conversion of nitriles **49** and **50** to their corresponding diamidines **53** and **54** as shown in Scheme 12.



Scheme 12. The Garigipati reaction of nitriles **56** and **57**

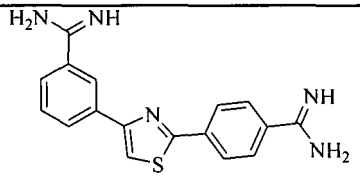
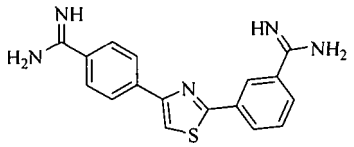
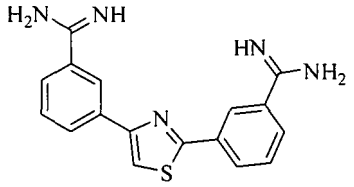
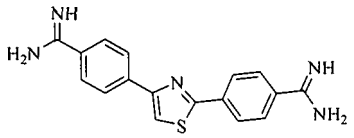
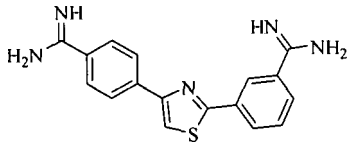
The alkylchloroaluminium amide reagent is generated from trimethyl aluminium ($(CH_3)_3Al$) and ammonium chloride ($NH_3.HCl$).[704] Addition of the aluminium amide to the nitrile forms an intermediate aluminium complex which is hydrolysed by water adsorbed on silica gel. When used in this case we found that although the low reaction time was an advantage and fewer side products were formed, the low solubility of the nitrile in toluene led to lower yields (22 %) than those obtained using Pinner methodology. Another route to the formation of amidines involves amidoxime reduction, a method used by Anbazhagan and co-workers for a range of amidoxime containing molecules.[705] Amidoximes were generated from the nitrile precursor using potassium *tert*-butoxide and hydroxylamine hydrochloride in dimethylsulfoxide[450] as shown in Scheme 13.



Scheme 13. Alternative route to the amidine *via* reduction of amidoxime **56**

We attempted the direct reduction of amidoxime **56** using catalytic transfer hydrogenation with palladium on carbon in ammonium formate and acetic acid. Although this reaction has been reported previously for biphenyl heterocyles,[415,705] thiazole amidoxime **56** was not affected. Due to the low reaction yield obtained for the Garigipati reaction and the failure of the amidoxime reduction, the Pinner reaction was used for the generation of all other 2,4-diphenylthiazole diamidines **51**, **52** and **55** as reported in Table 7.

Table 7. Structures of Targets

Comp No.	Structure	Yield (%)	Formula weight	H bond acceptors	H bond donors	Log P
58	 . 2TFA	22	$C_{17}H_{15}N_5S$ 321.40	5	4	3.21
59	 . 2TFA . H_2O	31	$C_{17}H_{15}N_5S$ 321.40	5	4	3.21
60	 . 2TFA	22	$C_{17}H_{15}N_5S$ 321.40	5	4	3.21
61	 . 2TFA	33	$C_{17}H_{15}N_5S$ 321.40	5	4	3.21
62	 . 2HCl . 2 H_2O	52	$C_{17}H_{15}N_5S$ 321.40	5	4	3.21

3.10 *In-Vitro* Antiplasmodial Activity and Inhibition of Hemozoin Formation of Thiazole *Bis*-Aryl Diamidines

3.10.1 *In-Vitro* Parasitic Sensitivity

Diphenylthiazoles **51-54** were assessed for antimalarial activity *in vitro* against CQ sensitive *P.falciparum*. The compounds were evaluated for their ability to inhibit parasitic growth by 50 % (IC_{50}) the results of which are shown in Table 8.

Table 8. Activity of thiazoles *versus* 3D7 and DD2 strains.

Compound	*IC_{50} (nM) 3D7	*IC_{50} (nM) DD2
58	257 ± 28	107 ± 15
59	279 ± 41	127 ± 25
60	225 ± 32	ND
61	273 ± 19	ND
CQ[1]	7 ± 4	96 ± 17
PMD[2]	162 ± 41	81 ± 11

[1] = Chloroquine; [2] = Pentamidine * Calculated from an average of triplicate determinations

Thiazoles **58-61** were formulated as trifluoroacetate (TFA) salts due to their purification method (preparative HPLC).[706] These analogues exhibit moderate antiplasmodial activity from 225 to 279 nM *versus* CQ sensitive parasites. Clearly the position of the amidine functionality on the phenyl ring has an effect on the activity of these analogues although not to a great extent, the *meta-meta* analogue being the most potent against CQ sensitive parasites; the *meta-para* diamidine is the most active against the resistant strain. Interestingly, these analogues are unaffected by the CQ resistant mutation thereby exhibiting their greatest potency against CQ resistant parasites with an enhancement of activity over 2-fold for most analogues tested. That being said, we were disappointed with their moderate activity especially when considering the activity of the closely related molecule DB75 (15.5 nM *versus* CQ resistant K1[695] 75 nM *versus* DD2).

At this point we were concerned about the project, especially due to our previous disappointment with isoxazole and oxazole heterocycles. Our resolve was further tested by a publication from the Tidwell group regarding the antimalarial efficacy of 3,5-bis(4-amidinophenyl) isoxazoles. Tidwell and co-workers synthesised isoxazole compounds with enhanced potency against *P. falciparum* in comparison to DB75 however, the majority of these compounds had cytotoxic indices ranging between 10 and 120 times higher than DB75.[695]

HCl salts contribute over 50% of all basic pharmaceutical salts approved in the last twelve years by the FDA (US Food and Drugs Administration).[707] Furthermore the isoxazoles assessed by the Tidwell group were formulated as HCl salts. We therefore considered that the salt form could play a crucial role in the potency of these compounds and hence reformulated compound **62** as a HCl salt.

Table 9. Activity of thiazoles *versus* 3D7 and DD2 strains.

Compound	*IC_{50} (nM) 3D7	*IC_{50} (nM) DD2
59	225 ± 56	156 ± 25
62	11.3 ± 2.1	6.2 ± 1.3
CQ[1]	7 ± 4	96 ± 17
DB75[2]	153 ± 26	75 ± 7.7
PMD[3]	162 ± 41	81 ± 11

[1]=Chloroquine; [2]= Furamidine; [3]=Pentamidine *Obtained from an average of triplicate determinations

To our excitement, reformulation dramatically enhanced the antimalarial potency of the *para-meta* HCl analogue **62** giving IC_{50}s of 11.3 and 6.2 nM *versus* CQ sensitive and resistant parasites respectively thereby displaying enhanced activity against CQ resistant parasites. We have not looked into the reasoning for the counterion effect in detail. Presumably TFA associates to the amidine nitrogen with a higher affinity than HCl affecting the amidine-propionate interaction or the solubilisation of the compound.

3.10.2 Inhibition of Hemozoin Formation

Earlier studies within this thesis have shown that PMD and propamidine inhibit the *in vitro* formation of hemozoin in line with their respective antiplasmodial activity. In addition, the Bray group have shown that PMD binds to FPIX in the test tube giving a pronounced quenching of the FPIX Soret peak on addition of 1 molar equivalent of PMD as shown in Figure 19.

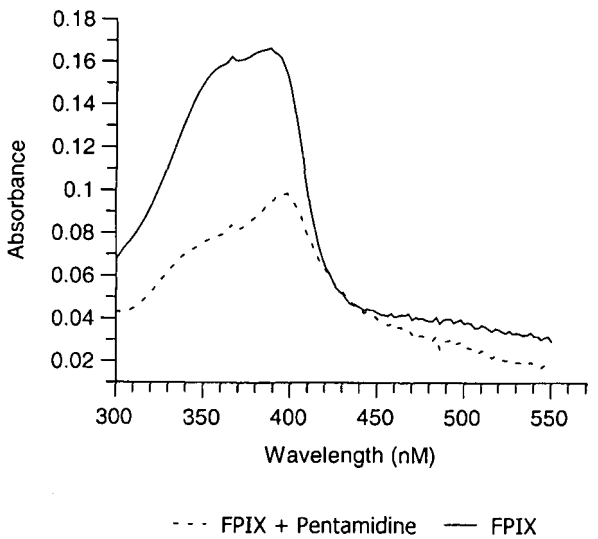


Figure 19. PMD quenching of FPIX Soret peak

We therefore assessed the ability of diamidine **62** to inhibit the formation of hemozoin using the inhibition assay of hemozoin formation from monomeric FPIX with ghost membranes *via* the same procedure employed for the PMD analogues assessed previously. Thiazole **62** was shown to be inhibit the formation of hemozoin *in vitro* when incubated with erythrocyte ghost membranes in a mode similar to CQ and PMD as shown in Figure 20.

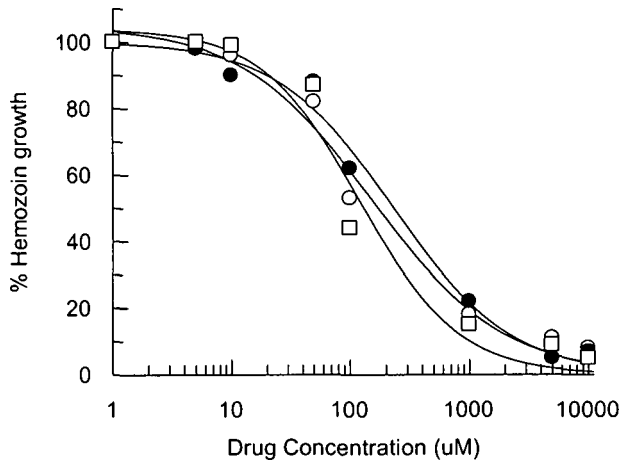


Figure 20. *In Vitro* inhibition of hemozoin formation from monomeric Ferriprotoporphyrin IX. (Generated from an average of triplicate determinations). Compounds are PMD (o), **62** (•), CQ (□)

However, the affinity of this binding interaction (230 μM) appears to be lower than PMD (152 μM) and CQ (121 μM) respectively. Clearly, this observation does not correlate with the *in vitro* potency of this compound. This suggests that this diamidine molecule may have additional specific uptake mechanisms compared with PMD. Alternatively, the key interaction may take place in the cytosolic/non-membrane component such that the ghost membrane assay is not a representative model for mechanism of action.

3.11 Molecular Modelling and Cytotoxicity

3.11.1 Molecular Modelling

To further investigate the heme binding properties of thiazole diamidine **62** *in silico* molecular modelling with heme was undertaken by Dr N. Berry (University *of* Liverpool) as depicted by the lowest energy conformation shown in Figure 21. Structurally the compound has heme binding properties, confirmed by the electronic interaction between the cationic amidines and anionic propionate groups.

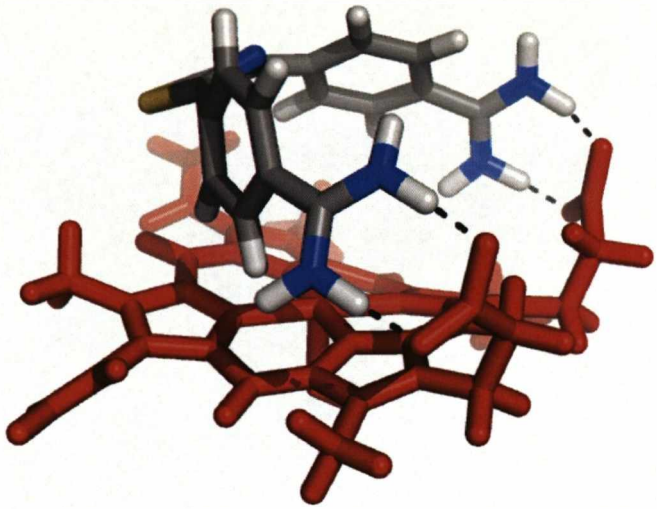


Figure 21. Low energy conformation of diamidine thiazole **61** binding to heme represented by; white = H, blue = N, yellow = S, grey = carbon. For method see section 5.1.6.

It is apparent from the conformation that the interaction is largely dependent on the diamidine-carboxylate contacts as oppose to propamidine where in addition to diamidine-carboxylate binding, the aromatic rings form a stacking interaction with the planar portion of the heme molecule, possibly explaining the reduced hemozoin inhibition activity profile produced by this molecule in comparison to PMD and CQ.

3.11.2 Cytotoxicity

Diamidine cytoxicity is widely reported,[310] frequently resulting in the termination of the development of compounds with potent antiplasmodial properties. In order to assess the toxic effects of biaryl thiazole **62** on the host cell, toxicity assays using hepatic Huh7 and Caco-2 intestinal cell lines were performed by A. Owen, University *of* Liverpool.[708,709] As shown graphically by Figures 22 and 23, the cytotoxicity of diamidine **62** was established to be less than DB75 against hepatic and intestinal cell lines with a promising therapeutic index of approximately 10,000.

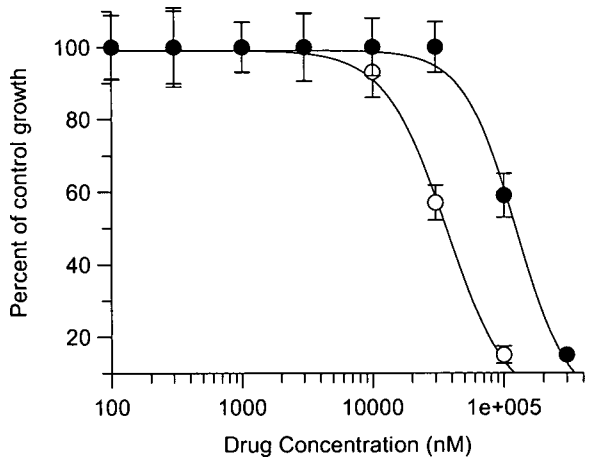


Figure 22. Cytotoxicity of thiazole **62** against hepatic cell lines (Huh7). Compounds are DB75 (o), **62** (•)

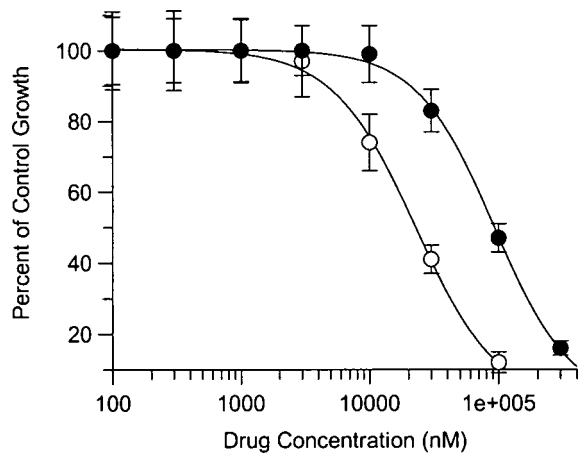


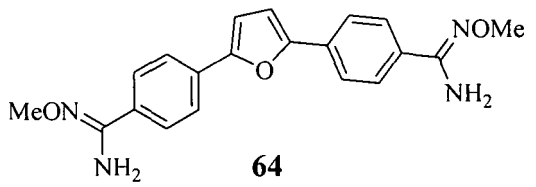
Figure 23. Cytotoxicity of thiazole **62** against intestinal cell lines (Caco-2). Compounds are DB75 (o), **62** (•)

Encouraged by the high potency and low cytotoxic potential of analogue **62**, we discussed a prodrug approach using this compound. We considered the re-synthesis of all analogues to assess their viability as HCl salts, particularly compounds **58** and **60** since they possessed enhanced activity against CQ resistant parasites and/or sensitive parasites compared to **52**. Accounting for the time it would take for their re-synthesis and re-assessment of antiplasmodial efficacy, we moved forward with analogue **55** as we were keen to explore the *in vivo* efficacy of appropriate prodrugs.

3.12 Thiazole Prodrugs

3.12.1 Rationale and Prior Art

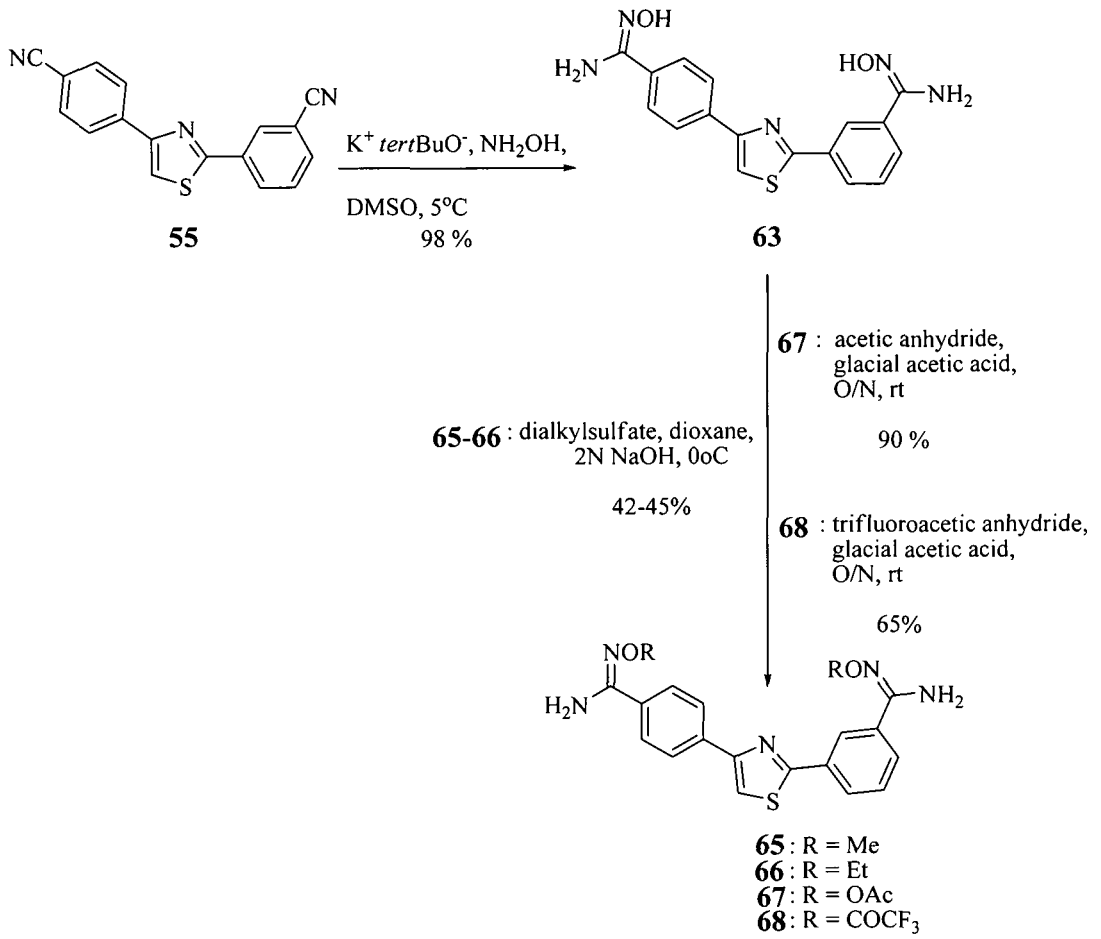
Diamidine compounds have a long history in the treatment of infectious disease particularly protozoan infections. However, their clinical deployment has been restricted due to problems associated with poor bioavailability, toxicity and the route of administration of the charged drug. Diamidines have long been known to possess the ability to suppress the growth of the *plasmodium* malaria parasite; however drugs with better physicochemical profiles, such as CQ and amodiaquine, became the principal compounds for prophylaxis. The efficacy and kinetics of pharmacologically active compounds are a function of their ADME properties. Due to a high pKa, the amidine moiety ionises readily within the acid milieu of the stomach and thus drugs such as PMD become inactive on oral administration and are therefore administered intravenously. A prodrug approach depressing the ability of the amidine functionality to protonate at physiological pH would therefore provide a route to the enhancement of oral potency. Such a strategy led to the development of the orally active DB75 prodrug DB289 **64**.



64

We therefore commenced the synthesis and assessment of antimalarial activity of novel diamidine prodrugs possessing a thiazole linker.

3.12.2 The Synthesis of 2,4-Diphenylthiazole Prodrugs



Scheme 14. Formation of thiazole prodrugs

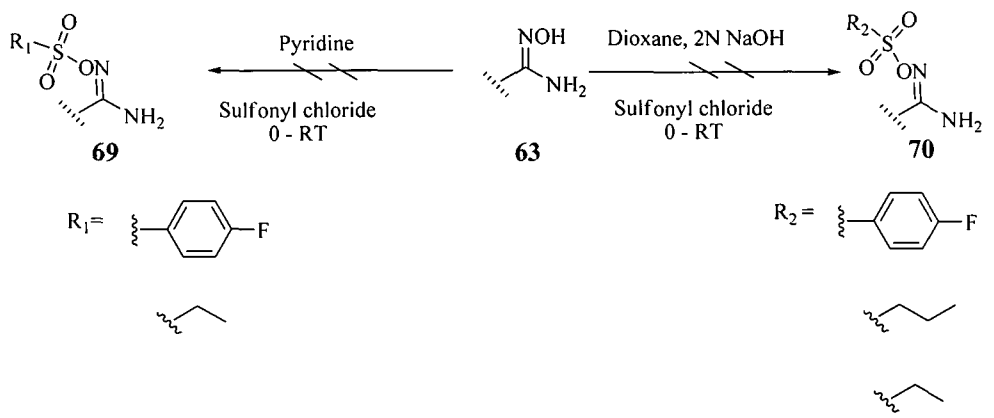
Prodrugs **63** and **65-72** were formed from the key intermediate dinitrile **55**. Amidoxime prodrug **63** was formed in excellent yield by the nucleophilic substitution reaction between dinitrile and hydroxylamine hydrochloride mediated by potassium *tert*-butoxide. Compounds **65** and **66** were formed from amidoxime **63** using alkylation methodology employing the appropriate alky sulphate in dioxane to give the alkylated product in typically 42-45% yield after purification by column chromatography.[450] This reaction was attempted several times before success was attained due to issues with the extraction of the product from the aqueous media. Pyridine was employed as an alternate base in an attempt to improve the yield of the reaction and ease of

product isolation however, use of pyridine led to a large decrease in the yield of reaction and thus NaOH was re-employed. Finally, acetate prodrug **67** and trifluoroacetate **68** were synthesised from a mixture of glacial acetic acid and acetic anhydride furnishing the title compound in an 89% and 79 % yields respectively. Table 10 shows the yield of formation and properties relating to each prodrug. Melting points are included since depression of melting point is a property used to ascertain prodrug qualities.

Table 10. Properties of thiazole prodrugs and parent compound

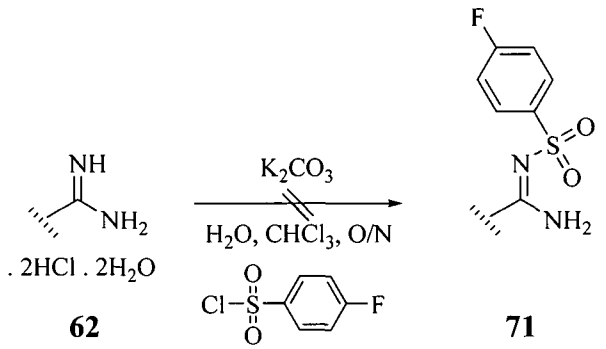
Compound Number	Pro-moiety	Yield / %	pKa	Log P	Melting Point / °C
63	NOH	98	8-9	3.55	218-220
65	NOMe	42	-	4.07	175-176
66	NOEt	45	-	4.75	145
67	NOAc	89	-	-	193
68	$NOCOCF_3$	79	-	-	197
Parent Drug	-	52	12-13	3.21	370

The synthesis of sulfonyl pro-analogues **69** and **70** was also attempted however, despite variation of the reaction conditions *via* variation of the base, the amidoxime was unaffected.[710]



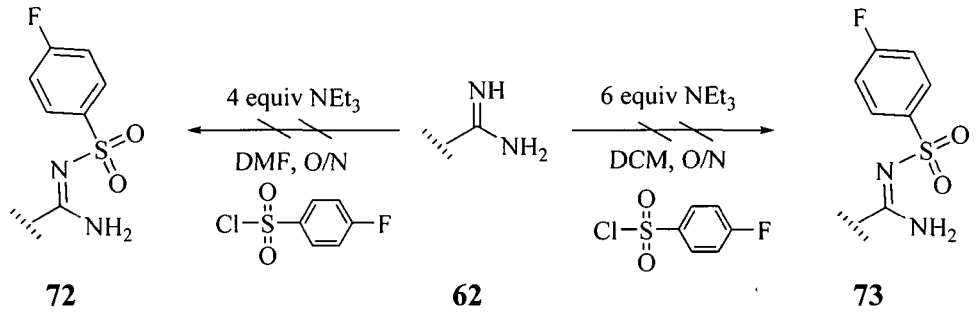
Scheme 15. Synthesis of Sulfonyl Prodrugs

Alternative chemical derivitisation approaches were attempted *via* the amidine free base using potassium carbonate in a mixture of DCM and water however this resulted in recovery of the starting material.[711] This reaction was also attempted *via* the amidine hydrochloride salt again using potassium carbonate and the appropriate sulfonyl chloride. Although this reaction has been reported previously for amidines, in this instance the reaction was unaffected.



Scheme 16. Synthetic Route to Sulfonyl Prodrugs

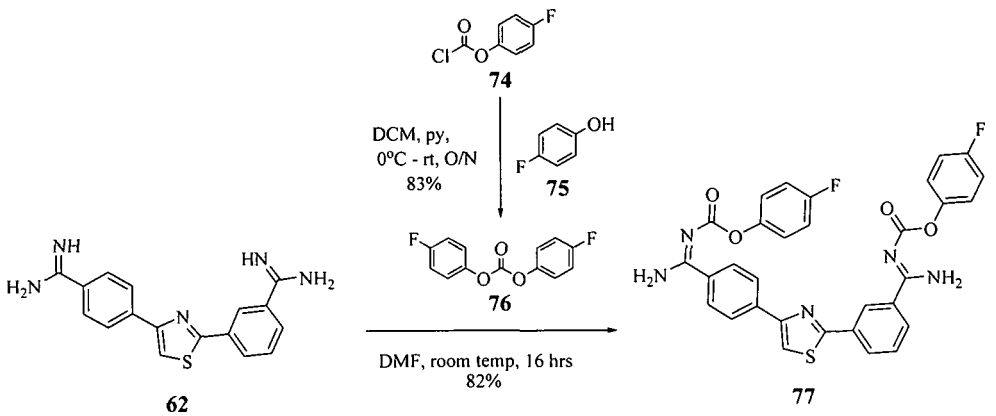
The reaction was therefore attempted using the amidine free base in the presence of triethylamine,[712] although consumption of the starting material was observed, the product formed was impure with a poor solubility profile thereby making its purification difficult.



Scheme 17. Synthetic routes to sulfonyl prodrugs from the diamidine free base **62**

Bis phenylcarbonates have been used as prodrugs to form protected amidines in good yields, thus we applied their methodology to the thiazole analogue.[496] Firstly the diamidine hydrochloride salt was converted to its free base, after which diamidine **62** was reacted with bis(4-fluorophenyl)carbonate **76** generated by reaction of 4-fluorophenyl chloroformate **74** and 4-fluorophenol **75** in DCM and pyridine, purification of **76** by column chromatography furnishing

the product in a yield of 83%. Carbonate **76** was then reacted with the amidine free base in DMF to give prodrug **77**. However due to issues with the purification of compound **77** related to its poor solubility profile in organic solvents this chemistry is ongoing.



Scheme 18. Alternative prodrug strategy

We assessed the *in vitro* antiplasmodial activity of these compounds and can confirm that they are indeed inactive with IC_{50}s > 1000 nM as expected.

3.13 Conclusion and Future Work

In conclusion, we have developed a series of novel diamidines that show moderate to excellent activity against *P. falciparum* strains *in vitro* dependent on the salt counterion. The activity of 2,4-diphenylthiazole diamidines is enhanced by mutant alleles of *Pf*mdr1 and *Pf*crt thereby surpassing the CQ resistance pathway. Furthermore, thiazole **62** is approximately 10-fold more active than DB75 against *P.falciparum in vitro* as shown by IC_{50}s of 6.2 and 75 nM respectively against the DD2 CQ resistant strain. This is important since it should allow for their novel application in combination chemotherapy where the diamidine moiety becomes more effective as resistance develops. With respect to mechanism of action, it is believed that diamidine compounds can bind to heme in a similar manner to the 4-aminoquinolines and maintain toxic monomeric heme within the parasite. Inhibition of hemozoin detoxification within the parasite as a mode of action for diamidines is further suggested by the observation that diamidines inhibit hemozoin formation *in vitro* with similar efficacy to CQ. In addition, compound **62** is effective

against CQ resistant *P. falciparum* with potency greater than the diamidine counterpart DB75 whose prodrug DB289 is currently in phase III clinical trials as a new orally active candidate drug to treat first-stage human African trypanosomiasis.[713] Furthermore, toxicity data established a favourable therapeutic index for thiazole **62** of 10,000.

An explanation for the failure to form the dinitrile isoxazole analogues in our hands is difficult to comprehend. Tidwell and co-workers synthesised isoxazole derivatives using CuCN in refluxing DMF to form the diycano product in yields from 21 – 69% from the dibromo precursor. For completion we repeated their methodology, again finding that the *mono* nitrile was formed alongside degradation products. Nonetheless, this is essentially the beginning of our investigations into the thiazole template. The chemistry prior to the cyanation step is facile forming the dibromo template in good yields without purification issues. Furthermore, thiazole chemistry circumvents the hurdles placed at every step of the synthetic route associated with isoxazole chemistry.

Future work and indeed work in-progress primarily involves a full assessment of *in vivo* oral bioavailability, antimalarial potency and pharmacokinetics of thiazole prodrugs using the *P. vinckei* rat model. Further investigations into the importance of hematin binding for activity by assessing the effect of inhibitors of haemoglobin digestion will also be undertaken. In addition, interactions with parasite lines of known drug resistance will be assessed in order to generate a profile of the probable resistance and/or cross resistance pattern of thiazole diamidines. Preclinical cell toxicity, genotoxicity and mutagenesis studies will also be performed.

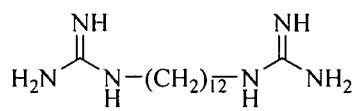
3.14 Alternative Dications

3.14.1 Project Aims

As well as preparing a small library of diamidine based heterocycles, we wished to expand upon this area through the study of alternative dications and heme binding templates. Our aims were to observe the effect of pKa on antiplasmodial activity by increasing the pKa of the dication. In addition to this, we further deviated from our previous studies by employing a fused ring system as oppose to one linked through a heterocycle.

3.14.2 Rationale and Prior Art

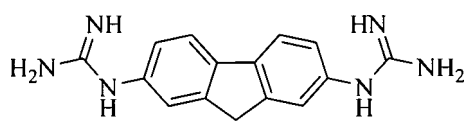
The antiprotozoal properties of diguanidines are known.[152,220-224,244,714,715] Calas and co-workers developed alkyl diguanidine **78**, a potent antimalarial with subnanomolar activity against a Nigerian strain of *P. falciparum*, found to exert an early effect on phosphatidylcholine (PC) biosynthesis.[152]



78

IC_{50} = 0.35 nM

In addition to this, using the fused fluorene ring system, Arafa *et al.* developed diguanidine **79** showing potent *in vitro* antiplasmodial activity against the CQ resistant K1 *P. falciparum* strain.[224]



79

IC_{50} = 2.3 nM

Our objective was to expand upon the studies of Arafa *et al.* by investigating the tolerance of a lumefantrine type template and a prodrug approach with respect to antimalarial activity.

3.14.3 Molecular Design

Molecules acting against the hematin crystallisation process have a long history within antimalarial drug design. It is believed that these molecules interact with hematin preventing its conversion to non-toxic hemozoin. Typical heme binding molecules contain a flat planar ring structure in order to form a π-π stacking interaction with the planar portion of heme; the fluorene unit possess such a ring structure. As previously discussed, the uniplanarity of fluorene has been debated. However, incorporation of the benzylidene unit at the fluorene methyl bridge can further enforce uniplanarity across the structure *via* sp^2 hybridisation. Furthermore, substitution at the phenyl ring allows variation of the lipophillic character of the molecule as shown in Figure 24.

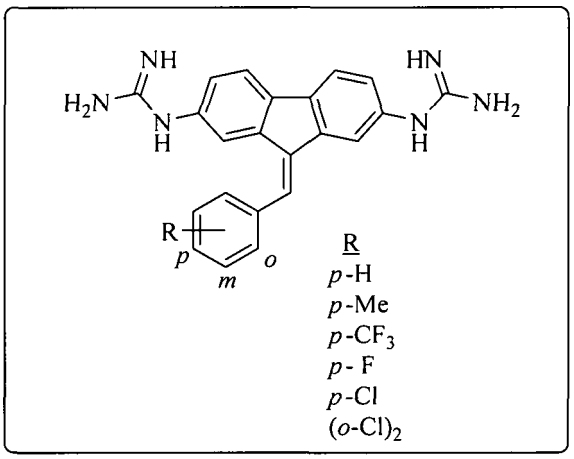


Figure 24. Diguanidine Template 1

Finally, incorporation of the *bis*-guanidine functions at the 2- and 7- positions enables binding to the anionic propionate groups, accumulation at vacuolar pH and the possibility of specificity for host cells *via* the new permeability pathway. However, the oral bioavailability of these molecules is subject to the highly basic nature of the guanidine moiety. Depression of the basicity of the guanidine moiety can be achieved by the attachment of a pro-moiety as represented by template 2, Figure 25.

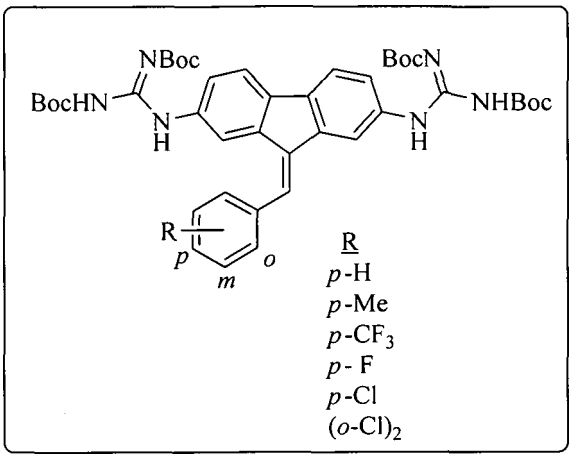
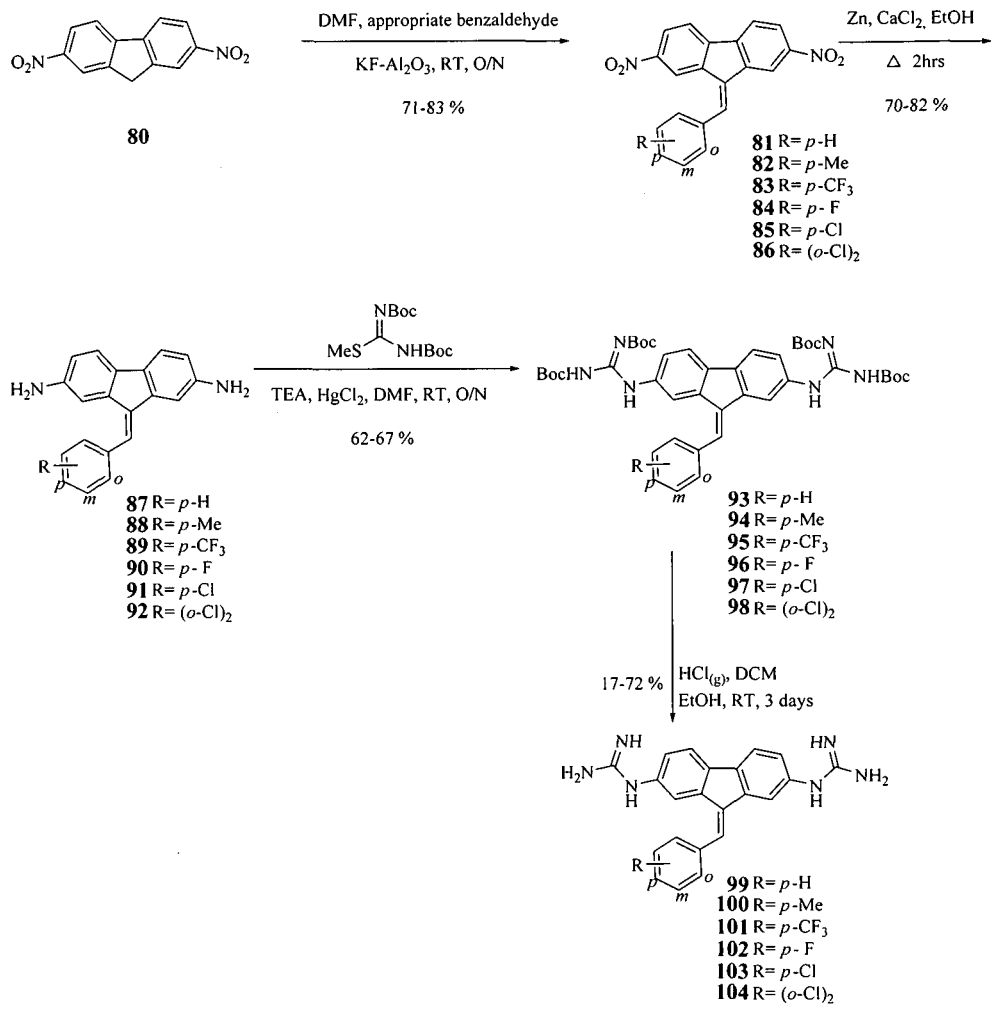


Figure 25. Boc-Diguanidine Template 2

3.14 Preparation of Fluorene Diguanidines

Due to the applications for extended π-electron systems, methods for the generation of 9-substituted fluorenes are widespread in the literature, particularly regarding benzylidene formation.[716-719] Substituted diguanidines **87-92** were synthesised from commercially available 2,7-dinitrofluorene as depicted in Scheme 19.

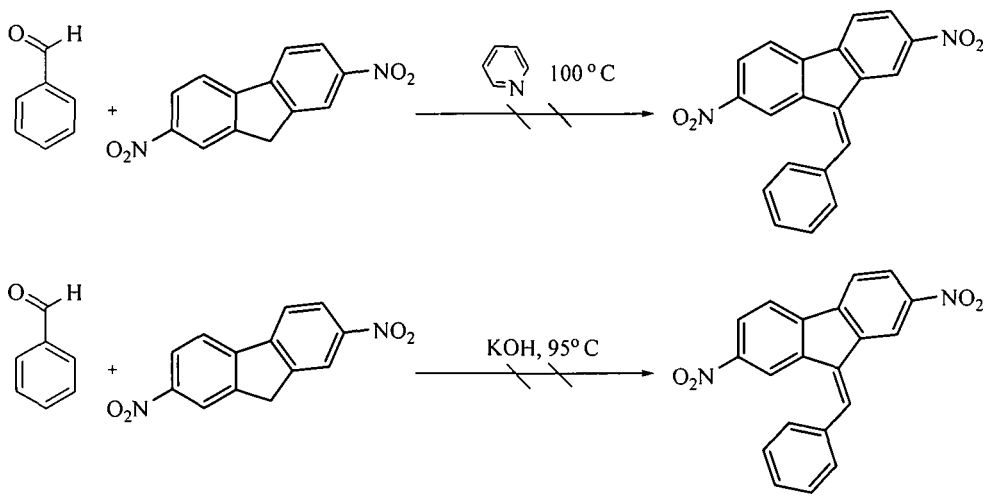


Scheme 19. Synthetic Route to Benzylidene Diguanidines

The first step of the synthesis involved the condensation of the appropriate benzaldehyde and dinitrofluorene **80**. In the presence of alumina supported potassium fluoride (KF-Al_2O_3) compound **80** was stirred at room temperature overnight to give compounds **81-86** in moderate yields.[720] It should however be noted that formation of the desired material was achieved after optimisation of the procedure. Lu *et al.* condensed fluorene with aromatic aldehydes using KF-Al_2O_3 in DMF at 150 °C to give fluorene benzylidenes in yields of 44-90%.[720] However, when attempting this reaction with 2,7-dinitrofluorene for the formation of benzylidenes **81** and **82** decomposition products were obtained, presumably due to the increased activity of this molecule upon nitro-substitution. We achieved the conditions shown in Scheme 19 by firstly performing the reaction at room temperature in order to monitor its course by TLC. We found that after 17

hours, a single product was formed however upon removal of excess DMF under vacuum at 65 °C we observed decomposition of the material. Therefore the product was isolated by filtering the solid product/ KF-Al_2O_3 reaction mixture and washing the solid with chloroform. Though the yield of reaction is adequate (71-83%), due to the poor solubility of the benzylidene product, separation from solid KF-Al_2O_3 was difficult. We did however further optimise the yield of benzylidene formation by drying the KF-alumina reagent at 130 °C under vacuum for 7 hrs prior to use.[721]

We also screened alternative procedures for the generation of benzylidenes. By heating dinitrofluorene **80** and benzaldehyde at 150°C in DMF we attempted the formation of fluorenebenzylidene **81**. However, in our hands, degradation occurred. We therefore assessed a base strategy facilitating the use of milder conditions. Using the methodology of Mysyk *et al.* and Plater *et al.* we employed pyridine[722] and potassium hydroxide[723] respectively as shown in Scheme 20.

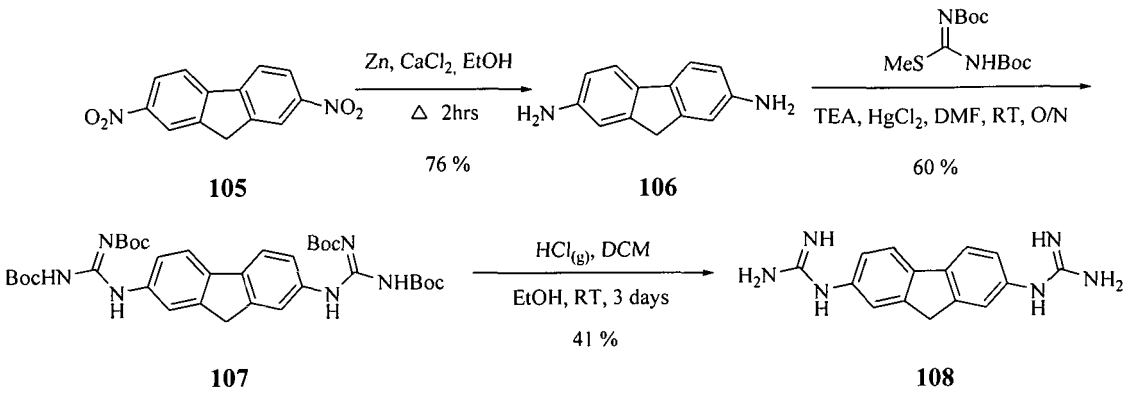


Scheme 20. Benzylidene Formation

In addition to these bases sodium ethoxide was also used however, degradation products were observed with all three bases respectively.

Following benzylidene formation, the nitro groups were reduced using zinc dust and calcium chloride as shown in Scheme 19.[724] Reduction of the nitro moieties was not without its problems,

compounded by the low solubility of the benzylidene starting material. We found that use of zinc dust, calcium chloride and EtOH markedly elevated the yield of reaction by enhancing the solubility of the starting material giving diaminobenzylidenes **87-92** in yields ranging from 70-82% after purification by column chromatography. This procedure was also applied to the formation of diaminofluorene **106** as shown in Scheme 21.

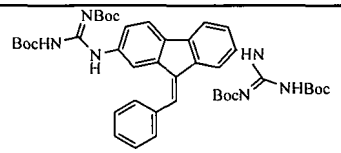
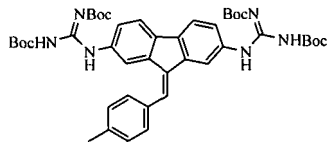
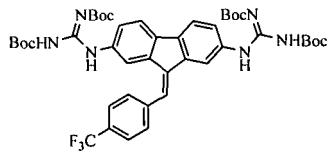
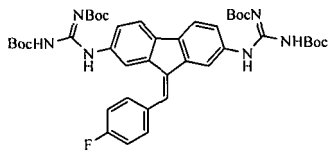
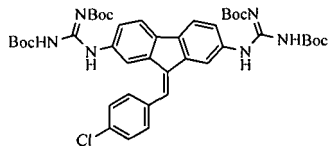
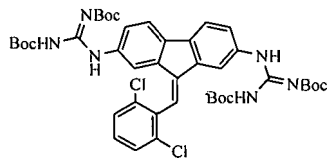
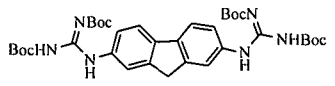


Scheme 21. Synthetic Route to Fluorene Diguanidine **108**

Initially we attempted the reduction using tin and hydrochloric acid, the intense effervescence of the reaction mixture upon heating however made this method unsuitable.[725] Ammonium formate and zinc in refluxing methanol was also used for the reduction, however the yield of product was low (20%) and the product proved difficult to purify.[726]

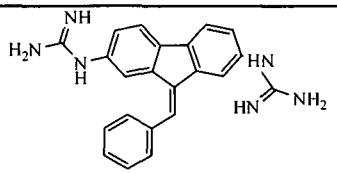
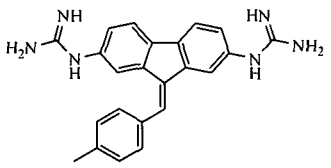
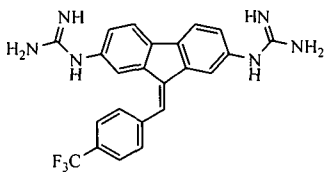
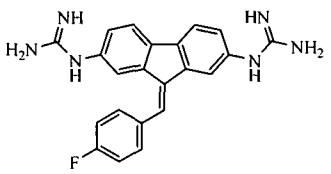
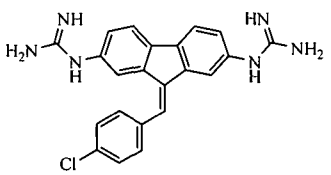
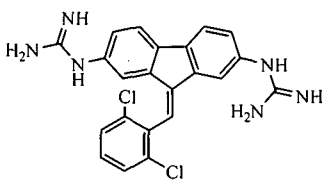
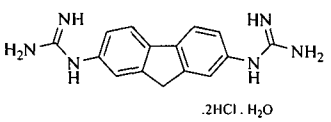
The conversion of *bis*-amines **106, 87-92** to their Boc-protected guanidines as shown in Scheme 19, proceeded with fewer issues. Using triethylamine, mercury (II) chloride and the methyl thiopseudourea derivative in DMF, Boc protected guanidines **107, 93-98** were formed in 60-67% yield as shown in Table 11 after purification by column chromatography.[727]

Table 11. Formation of Boc-protected diguanidine prodrugs

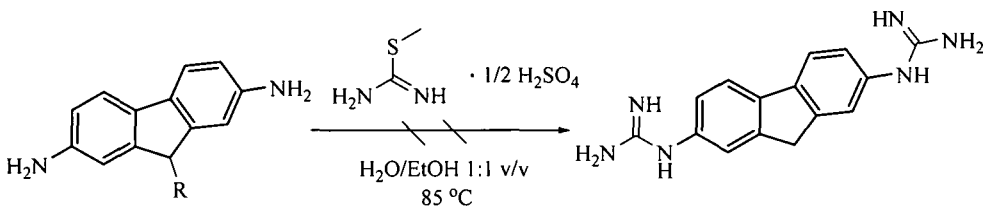
Comp No.	Structure	Yield (%)	FW	H bond acceptors	H bond donors
93		65	$C_{42}H_{52}N_6O_8$ 768.99	14	4
94		62	$C_{43}H_{54}N_6O_8$ 782.92	14	4
95		65	$C_{43}H_{51}F_3N_6O_8$ 836.90	14	4
96		65	$C_{42}H_{51}FN_6O_8$ 786.89	14	4
97		67	$C_{42}H_{51}ClN_6O_8$ 803.34	14	4
98		67	$C_{42}H_{50}Cl_2N_6O_8$ 837.79	14	4
107		60	$C_{35}H_{48}N_6O_8$ 680.79	14	4

Boc-protected analogues were converted to diguanidines **108**, **99-104** by bubbling HCl gas through a DCM/EtOH mixture to saturation and sealing the vessel at room temperature over 3 days, to form the product in yields from 17-72% as shown in Table 12.[224,727]

Table 12. Formation of Diguanidines

Comp No.	Structure	Yield (%)	FW	H bond acceptors	H bond donors
99		71	$C_{22}H_{20}N_6$ 368.43	6	6
100		17	$C_{23}H_{22}N_6$ 382.46	6	6
101		58	$C_{23}H_{19}F_3N_6$ 436.43	6	6
102		72	$C_{22}H_{19}FN_6$ 386.42	6	6
103		70	$C_{22}H_{19}ClN_6$ 402.88	6	6
104		37	$C_{22}H_{18}Cl_2N_6$ 437.32	6	6
108	 .2HCl . H_2O	41	$C_{15}H_{20}Cl_2N_6O$ 371.26	6	6

Due to the moderate yields for these reactions and a requirement for gaseous HCl, we assessed alternative synthetic routes. The Rathke reaction involves nucleophilic attack by the amine moiety on the amidine derivative 2-methyl-2-thiopseudourea to yield a guanidine and has been used successfully for the generation of phenylguanidines.[728] Unfortunately, when attempted in this case product formation was not observed.



R = H; **106**
R = Benzylidene **87**

Scheme 22. The Rathke reaction

3.16 *In-Vitro* Antiplasmodial Activity of Fluorene Diguanidines

3.16.1 *In-Vitro* Parasitic Sensitivity

Seven diguanidines **108** and **99-104** were assessed for antimalarial activity *in vitro* against CQ sensitive and CQ resistant *P.falciparum* strains 3D7 and DD2 respectively, the results of which are shown in Table 13.

Table 13. Activity of diguanidines **108** and **99-104**

Compound	*IC_{50} / nM DD2	*IC_{50} / nM 3D7
99	703	207
100	>1000	291
101	829	275
102	768	204
103	842	219
104	802	231
108	>1000	201
CQ	64	8
PMD	105	106

*SD within 10% taken from an average of triplicate determinations

Against CQ resistant parasites, fluorene and fluorenebenzylidene diguanidines show high nM activity. The most potent analogue is benzylidene **99** with an IC_{50} of 703 nM, the least potent being diguanidines **108** and **100** with IC_{50}s >1000 nM. These analogues were found to be more potent against CQ sensitive analogues as their activity improved markedly against the 3D7 isolate. This can be observed most notably for diguanidines **100** and **108** with activity ranging from 1000 nM to 291 and 201 nM respectively against the 3D7 strain. Moreover fluorene diguanidine **108** is the most potent of the 3D7 series followed closely by compound **102** (204 nM), the *p*-fluoro analogue.

It appears that benzylidene formation does not affect activity to a great extent as shown by the non-substituted fluorene **108** and benzylidene **99** with IC_{50}s of 201 and 207 nM respectively against CQ sensitive isolates. This is not however the case for CQ resistant isolates as shown by IC_{50}s of >1000 and 703 nM for the same analogues respectively. Furthermore, heteroatom substitution at the phenyl ring did not appear to have a positive affect on compound activity. However, while fluorine substitution gave the more potent analogue **102**, trifluoromethyl substitution gave an analogue with reduced activity from 204 to 275 nM for compounds **102** and **101** respectively. Boc prodrugs **107** and **93-98** were also tested for antimalarial activity against *P. falciparum* as shown in Table 14.

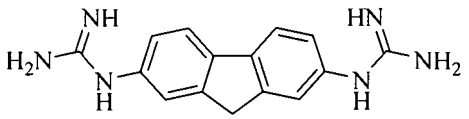
Table 14. Activity of Boc prodrugs

Compound	*IC_{50} / nM 3D7
93	156
94	[a]IS
95	183
96	119
97	142
98	87
107	[a]IS
CQ	6
PMD	109

[a]IS = Insoluble; * SD within 10% taken from an average of triplicate determinations

Interestingly, the Boc prodrugs were more active than their diguanidine counterparts against CQ sensitive isolates. The most potent analogue **98**, the 2,6-dichlorobenzylidene analogue, gave an IC_{50} of 87 nM compared to 231 nM for the parent drug. The least active compound of the series was **95**, the trifluoromethyl analogue gave an IC_{50} of 183 nM although this was still greater than its parent drug whose IC_{50} was 275 nM. The least potent analogue of the diguanidine series was methyl substituted fluorenebenzylidene **100** (291 nM) however, an IC_{50} was not generated for the Boc prodrug as this compound was only partially soluble in both DMSO and a mixture of EtOH

and water. Considering the activity of the previously assessed fluorene diguanidine **79**,[224] we are surprised at this result.



79

IC_{50} = 2.3 nM

It should however be noted that molecule **79** was assessed against the K1 CQ resistant strain of *plasmodium* while our compounds were assessed against DD2 since we have found cross contamination with the K1 strain to be a problem. Furthermore, differing strains can give rise to varying activity profiles. In addition, the diguanidine analogues proved difficult to solubilise since this form of aromatic system can aggregate readily thereby affecting solubility, a factor likely to contribute to the poor *in vitro* activity of the parent drugs. The increased pKa of the guanidine functionality compared to the amidine group may also affect its uptake into parasitised cells, possibly explaining why Boc prodrugs are more active than the parent drug since their uptake into infected erythrocytes is enhanced.

3.17 Conclusions and Future Work

We have synthesised a range of phenyl substituted fluorene diguanidines in moderate yields from commercially available 2,7-dinitrofluorene with a route to the Boc prodrug. When assessed for antimalarial activity, the parent compounds were poorly active *versus* CQ resistant parasites and moderately active against a CQ sensitive strain. However, when assessing the antimalarial activity of the parent diguanidine compounds, there were issues with the solubility of these compounds which may have played a role in their poor activity. Furthermore these compounds were not tested against the CQ resistant K1 strain, which may account for their low activity in comparison to compound **79**. The *in vitro* activity of Boc prodrugs was promising, thus a future assessment of their *in vivo* activity will give superior information regarding the chemotherapeutic potential of these compounds.

CHAPTER III

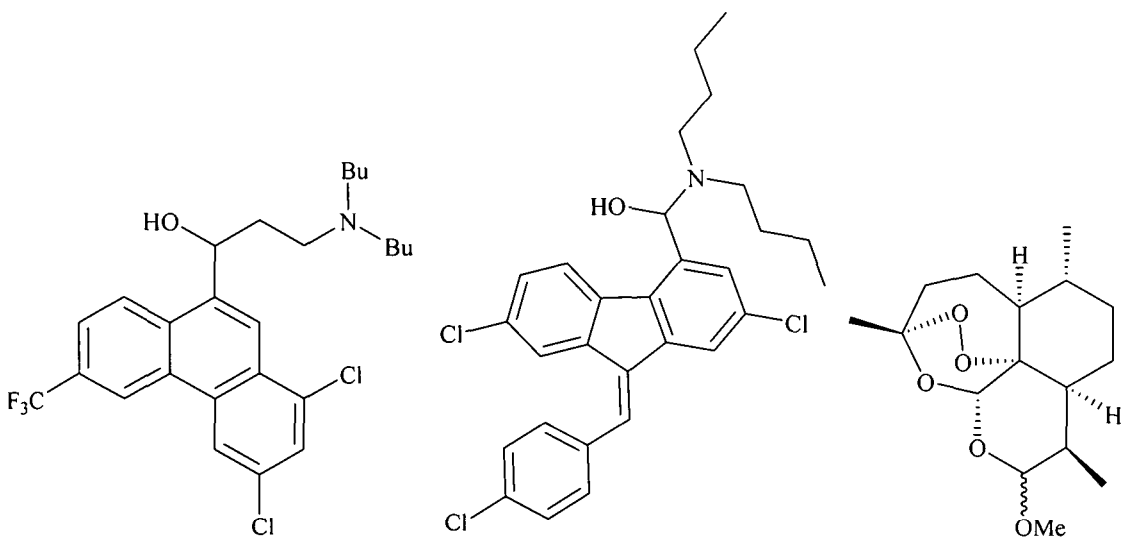
PART II

Results and Discussion – Mono-Cationic

3.18 Medicinal Chemistry of Amino-Alcohol Antimalarials

3.18.1 An Introduction to Lumefantrine ((E)-2-(dibutylamino)-1-(2,7-dichloro-9-(4-chloro-benzylidene)-9H-fluoren-4-yl)ethanol))

Lumefantrine (Lu, formerly Benflumetol, Figure 26) is a racemic fluorene methanol of the amino-alcohol antimalarial subclass that was synthesised and assessed for antimalarial activity by the Academy of Military Medical Science, Beijing, and registered in China in 1992. It is well tolerated in both adults and children when combined with Artemether (COARTEM®),[78] having a very low acute and subacute toxicity,[729,730] which has been confirmed by extensive cardiological investigations[652] with both enantiomers exhibiting the same blood schizontocidal activity. The mechanism by which the amino-alcohol class works is unclear although blood stage parasites and heme detoxification are inferred to be the target but since mefloquine, halofantrine (Figure 26), quinine and Lu have been shown to be poor inhibitors of β-hematin formation *in vitro*,[731] additional targets to the haemoglobin degradation pathway may possibly be involved.



Halofantrine (HF) **Lumefantrine (Lu)** **Artemether**

Figure 26. Structure of Halofantrine, Lumefantrine and Artemether

However, it has recently been shown that HF (halofantrine, Halfan®) co-ordinates to heme *via* three modes consisting of co-ordination to the central Fe(III) porphyrin centre through the HF aryl alcohol group, π-stacking between the phenanthrine ring and planar porphyrin system and intermolecular hydrogen bonding between the protonated nitrogen atom of HF and heme propionate residues. In addition, this propionate forms a second hydrogen bond with an adjacent Fe(III)PPIX propionate group as shown in Figure 27 a and b.[732] This provides a new model for the mechanism of action of HF, having implications for the possible mechanism of action for the amino-alcohol antimalarial subclass and the F-M hybrids discussed herein.

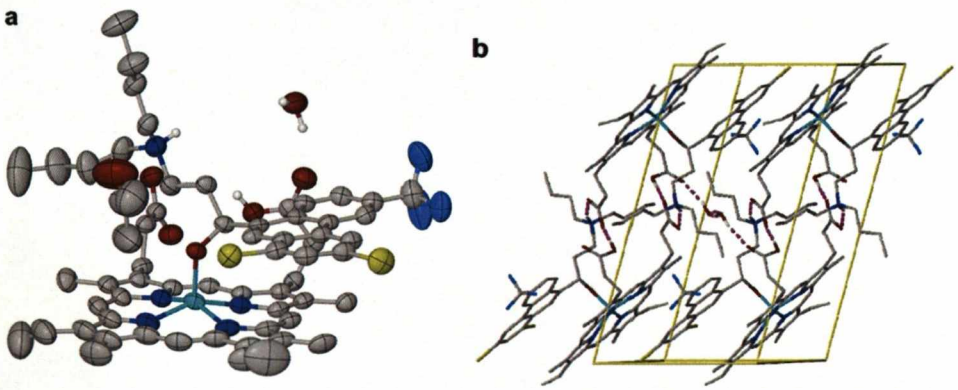


Figure 27. a) HF–Fe(III)PPIX crystal structure **b)** π-stacking and hydrogen bonding in the crystal[732]

There are several disadvantages associated with the use of Lu, including cost (related to the synthetic route)[733] and bioavailability which is dependent on co-administration with fat,[76,77,734,735] increasing approximately 16-fold[736] meaning that administration must be accompanied by food; thus absorption is variable, improving with recovery from malaria. This is a setback since most patients suffering from malaria suffer from anorexia, are unable to eat and experience emesis due to the nature of the infection.

Artemisinins were originally available as monotherapy; however if used as a single dose, treatment must be adhered to for no less than five, and typically seven days.[737] Since the treatment period is long, in practice compliance to these treatment regimens is low.[738] Additionally, short course monotherapy of artemisinin, artemether (Artme, Figure 26) and artesunate result in recrudescence.[739,740] Lu/Artme combinations display substantial synergism,[741] particularly in the IC_{90} and IC_{99} regions of laboratory models. This has been confirmed clinically

in the form of COARTEM® and RIAMET® (Novartis) for the treatment of *falciparum* malaria, within which it is the dominant component (20 mg Art, 120 mg Lu).[78,652,742] Since drug combinations have the potential to delay drug resistance the world health organization (WHO) recommends the use of fixed-dose combinations of antimalarial drugs,[743] most notably Artemesinin based combination therapies (ACTs) as they have been shown to aid treatment compliance thereby optimising clinical efficacy, avoiding recrudescence and developing resistance. Several countries (see Figure 28) including Kenya,[744] Burkina Faso[745] and Bangladesh[746] have changed their policy to encourage the use of ACTs with Uganda changing its policy in 2005 to incorporate Artme-Lu as the first-line antimalarial therapy and artesunate-ADQ as an alternative.[747] Artme-Lu is now more effective than sulfadoxime-pyrimethamine (S-P) for uncomplicated malaria in Nepal.[748]

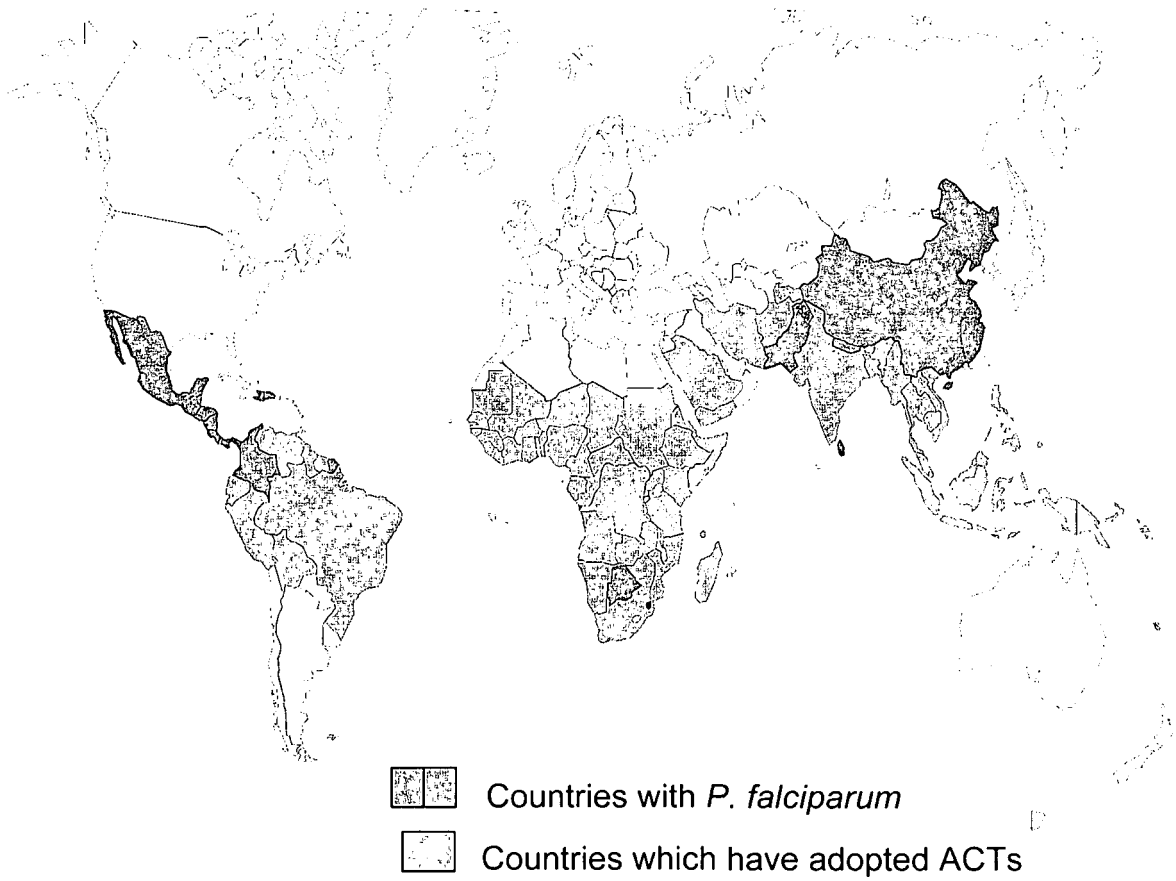


Figure 28. Countries that have adopted ACTs as National policy for the first-line treatment of uncomplicated falciparum malaria. Reproduced by kind permission of the Global Malaria Program, World Health Organisation[749]

At present, ACTs are the first-line favoured treatment for uncomplicated *falciparum* malaria. Resistance is emerging to combinations of artesunate/mefloquine thus becoming ineffective in some areas of the Thai-Cambodian border.[735] Therefore the development of alternative combinations is required. Artme has a schizontocidal effect superior to Lu and hence clears the bulk parasite mass thereby rapidly reducing parasitaemia. Due to its extended elimination half life, Lu (Artme ~ 1hr, Lu 4-6 days) is used within the combination to eradicate residual parasites with best cure rates at 99 % with a six-dose regimen given over five days.[652] Lu is structurally related to HF which has associated toxicity issues due to QTc (heart rate interval) prolongation. This protraction of ventricular repolarisation is significant, positively correlates to HF treatment and is therefore exposure dependent. Use of Lu-Artme combination therapy has been studied extensively showing that the QT interval is unchanged and no significant cardiovascular toxic effect of this type is observed *in vivo*.[72,73,750]

3.19 Medicinal Chemistry of Aminoquinoline Antimalarials

Erythrocytes are the cellular target for malarial protozoa. Host cell haemoglobin is used for the acquisition of cellular material essential for parasitic growth and development. The elucidation of the molecular structure of quinine (1854)[751] facilitated the exploitation of this process through the cessation of parasitic growth. Investigations into the total synthesis of quinine[752] led to the development of the 4-aminoquinoline subclass of antimalarials of which CQ and ADQ (amodiaquine) are members with quinine and mefloquine representing the quinoline-methanol subclass.

3.19.1 Mechanism of Aminoquinoline Antimalarial Activity: Chloroquine.

Haemoglobin degradation occurs within the parasitic food vacuole creating heme and globin protein, the later being utilised for amino acids (Figure 29). Redox active monomeric free heme - FPIX (also referred to as hemin) is toxic to the parasite. The parasite is unequipped to manage and degrade this toxic by-product resulting in a disposal problem, solved through a detoxification

pathway converting heme to a nontoxic polymer hemozoin the 4 aminoquinoline antimalarials (CQ and ADQ) work through inhibition of hemozoin formation by means of an interaction with hematin).[56,57,691,753]

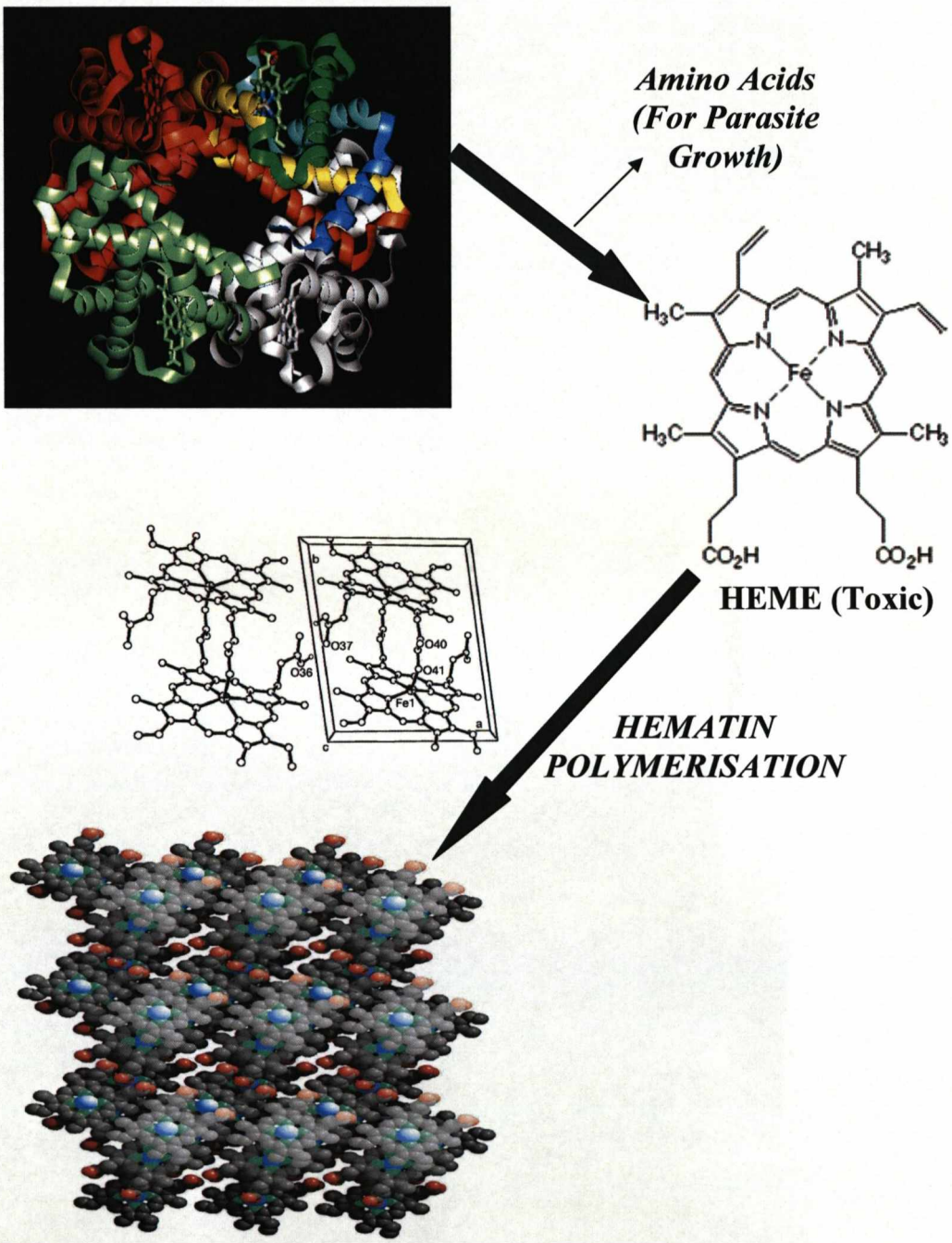


Figure 29. Degradation of haemoglobin and hematin polymerisation.[685]

Free heme and heme-CQ complexes kill parasites by inducing oxidative stress from the formation of reactive oxygen species (ROS) and the inhibition of heme binding proteins (glutathione, GSH), which may lead to peroxidation of parasite membrane lipids, damage of DNA, oxidation of proteins and finally parasite death as depicted in Figure 30.[754,755]

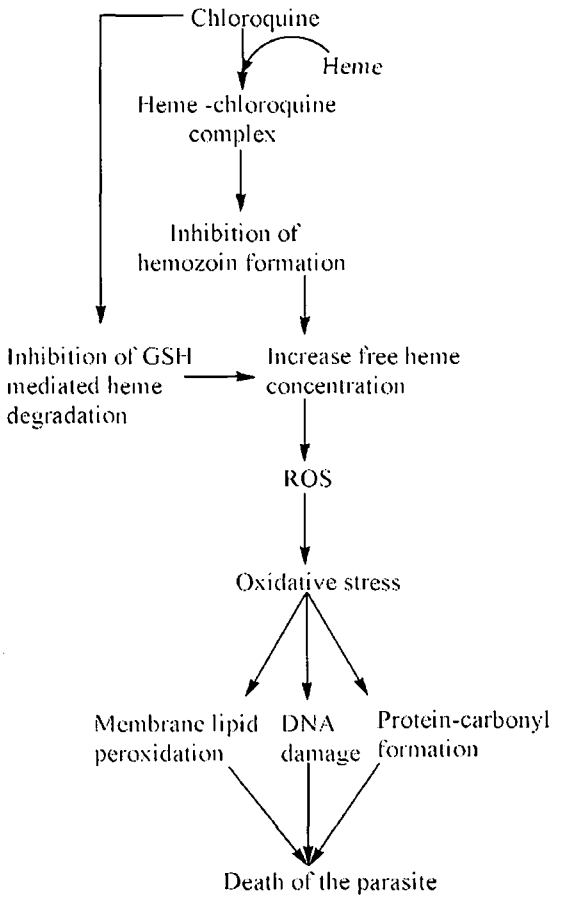


Figure30. Mechanism of antimalarial activity of chloroquine[755]

In the presence of free heme, CQ and quinidine associate with polymeric heme. It has been revealed that within parasite culture, blocking heme release with protease inhibitors is antagonistic to MQ (mefloquine) and CQ action.[55] The 4-aminoquinolines; CQ and ADQ (ADQ used for chemoprophylaxis rather than treatment), quinoline methanols; MQ and quinine are understood to be proficient in prevention of the formation of hemozoin due to their flat planar ring systems which permit interaction electronically with hematin through π–π stacking between monomeric hemin sheets. In addition the terminal nitrogen provides efficient accumulation into

the acidic parasite food vacuole. CQ, AQ, QN, MQ and HF all exert an effect at the erythrocytic stage of development. Haemoglobin degradation is believed to be part of their mechanism of action although the interactions between the various drug classes and hematin are fundamentally different as the more lipophillic quinoline methanols MQ and QN are not concentrated so extensively in the food vacuole and therefore it is likely they have additional/alternative modes of action.[56,756,757]

CQ can traverse the erythrocyte unprotonated as a diprotic weak base progressing down the pH gradient (Figure 31) to enter the food vacuole. Protonation within the acidic environment causes an accumulation of CQ 10,000-fold when compared to uninfected cells.[758] Furthermore, CQ resistant parasites accumulate less CQ than CQ sensitive ones.[52] CQ has such a wide range of pharmacological effects in addition to antimalarial activity[759-761] that the elucidation of the mechanism of action has been difficult and under debate.[762-765] Most theories could not explain the non-toxicity of CQ toward mammalian cells. However, CQ is active only against the erythrocytic stages of malaria parasites. Specifically, CQ acts during the stages of the intraerythrocytic cycle exclusively where the parasite is actively degrading haemoglobin.[766]

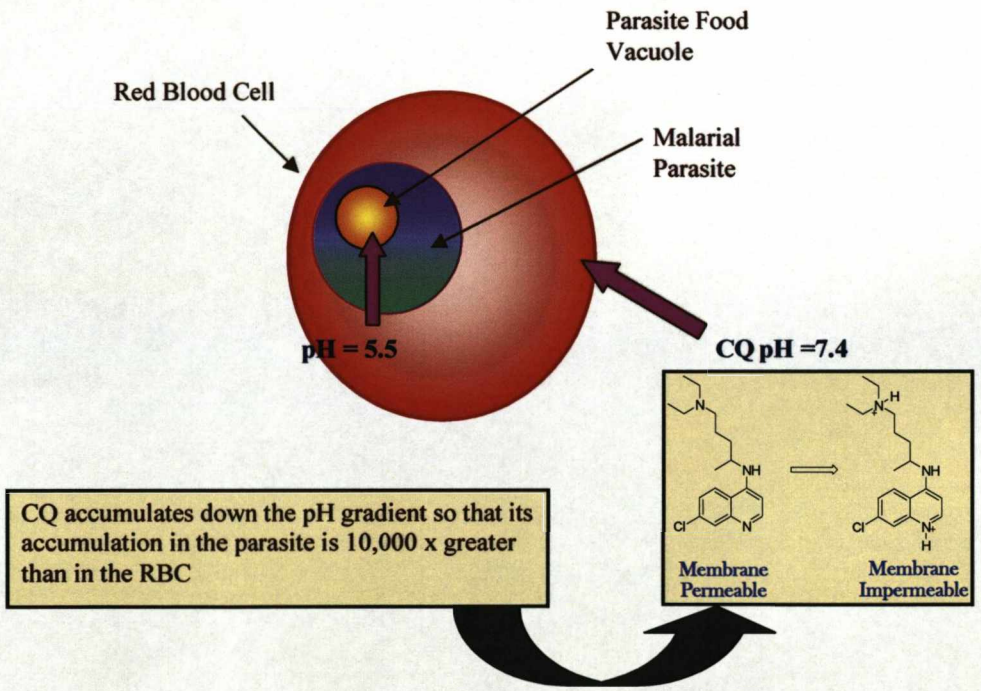


Figure 31. Accumulation of Chloroquine within a parasitised red blood cell.

3.20 Fluorene-Mannich Hybrids: Drug Design

3.20.1 Rationale

CQ is arguably one of the most important agents in the history of drug design however widespread resistance has limited its use, resulting in an urgent requirement for alternative chemotherapeutic agents. The haemoglobin degradation pathway is a well established target of antiplasmodial pharmacological intervention and novel chemical entities that are capable of complexing hematin provide an accessible route to circumvent the 4-aminoquinoline resistance mechanism. Knowledge of the critical features of CQ that facilitate its antimalarial efficacy and ability to achieve a high concentration in the food vacuole highlight ion-trapping and high affinity binding to hematin as key to the mechanism of action. Based on this knowledge it seems logical that a new class of antimalarials should be accessible by use of the fluorene unit as a hematin binding group in addition to a basic protonable side chain. Moreover fluorene is a known hematin binder present in the antimalarial compound Lu. Lu has issues associated with its clinical use due to inadequate bioavailability leading to a variation in treatment success dependent on the fitness of the individual.[77] Another setback to the use of Lu is the cost linked to its expensive synthesis[733] in addition to the fact that Lu is chiral, a property we wish to avoid.

The chemistry, medicinal chemistry and structural properties of fluorene were previously reviewed in order to introduce the fluorene unit. The structure of fluorene is of importance since there is a requirement for a flat planar structure in order to form a π-π stacking interaction with planar heme. Although some evidence points towards a flat fluorene molecule we have enhanced the planarity across the template by the introduction of a double bond at carbon-9. In addition to this, the rationale behind the design of Fluorene-Mannich (F-M) hybrids includes a protonable amino side chain for accumulation and a hydrogen bond donating group for possible interaction with the carboxylate residues of heme as described in Figure 32. Furthermore we have accomplished their synthesis using a three step modular process to an achiral template. When designing the compounds we were keen to avoid the use of diprotic analogues in an attempt to avoid the toxicity issues associated with non-specific cellular accumulation of antimalarials such as ADQ.[63]

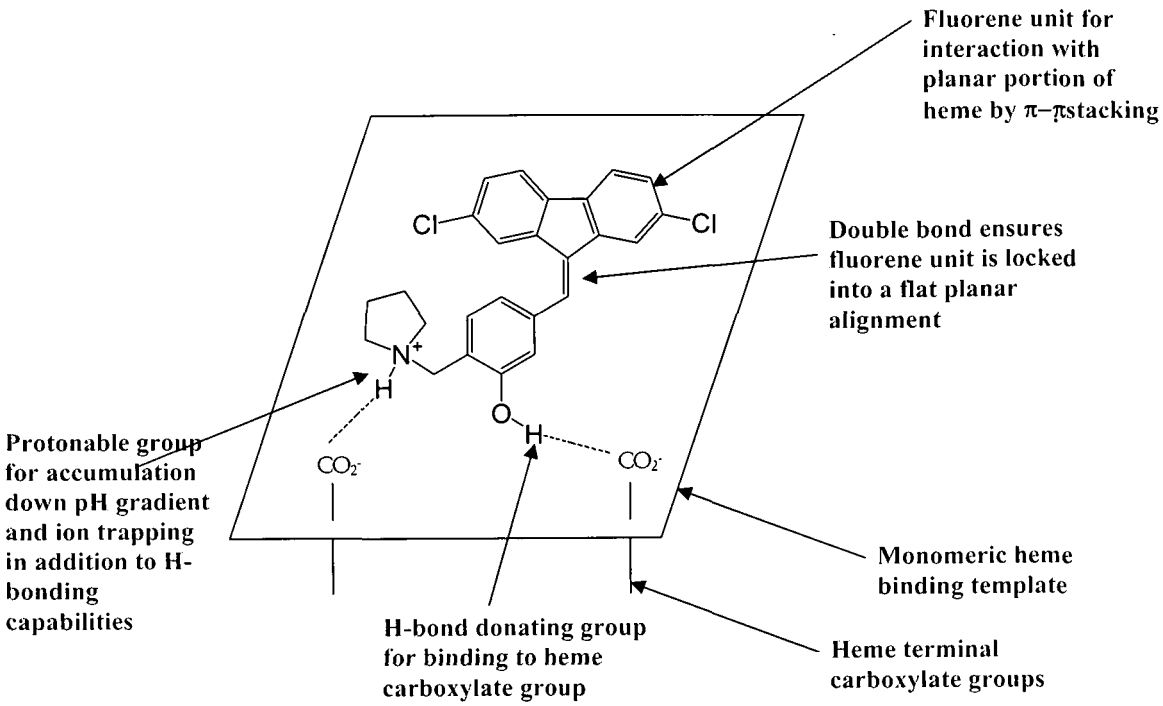
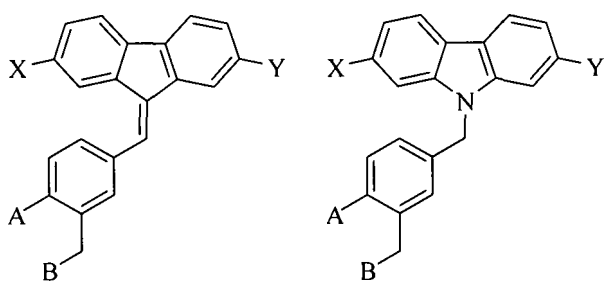


Figure 32. Fluorene-Mannich Hybrid Drug Design



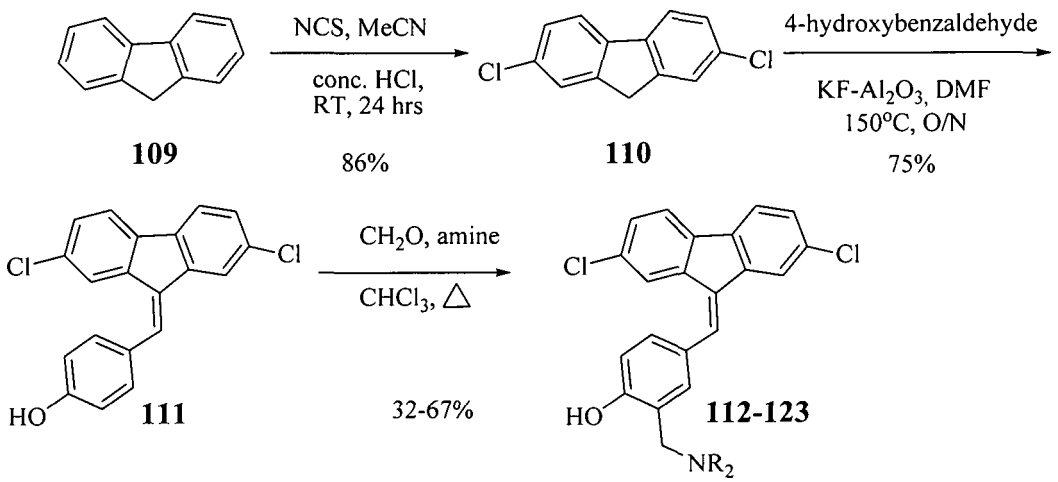
X = Cl; Y = Cl; A = OH; B = Amine

Chart 1. Structure of Fluorene-Mannich Hybrids

3.21 The Preparation of Fluorene-Mannich Hybrids

3.21.1 Chlorination of Fluorene

An examination of the reactivity of fluorene, in particular electrophilic substitution at the phenyl rings and carbon-9 led to the development of the synthetic route outlined in Schemes 23 and 24. When designing the process it was imperative to us that the synthetic route adhered to the challenges met within the pharmaceutical industry and medicinal chemistry, paying particular attention to process design: assessing both the volume and nature of waste produced, number of synthetic steps, chirality and cost of materials in a divergent route if possible.

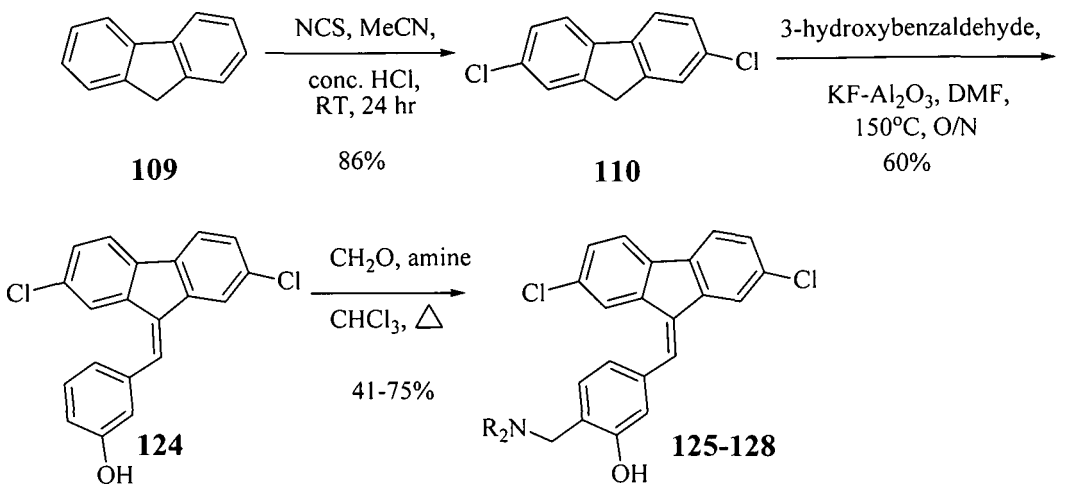


Scheme 23. Formation of 4-hydroxy analogues

Initially 2,7-dichlorofluorene was purchased from Aldrich. However we were keen to find an effective method for the conversion of fluorene to its dichloro adduct avoiding the use of methods that would lead to laborious separation and purification of the products, which are commonly *mono*, di and tri chlorinated, as experienced during the current method used for the synthesis of Lu. Beutler *et al.* (Novartis Pharma) use chlorine in acetic acid at 40°C generating *mono*, tri, 2,5 and 2,7 dichlorinated fluorene.[733]

An examination of the literature made it apparent that methods for the chlorination of fluorene in good yields were sparse and mainly involved gaseous chlorine and acetic acid. However, further

investigations revealed Perumattam's 1994 paper in relation to the alkylation and chlorination of fluorene which was applied here to affect the chlorination of fluorene using NCS in MeCN at room temperature.[570] Subsequent recrystallisation from MeOH furnished the 2,7-dichloro analogue in 90 % yield.



Scheme 24. Formation of 3-hydroxy analogues

3.21.2 Aldol Condensation

Substitution at position-9 was a key step in the formation of Fluorene-Mannich hybrids and was more facile for *para*-hydroxy aldehydes than *meta*. KF/Al_2O_3 in DMF at 150°C has been shown to be effective for the aldol condensation of fluorene with aromatic aldehydes.[720] We tried Lu's method[720] for the preparation of substituted guanidines (covered previously) finding that the reaction works best at room temperature. Consequently we tried to perform the reaction at room temperature however this resulted in a large presence of starting material. We then elevated the temperature to 150°C establishing that although the reaction generated the desired material, the yields were moderate. In an attempt to increase the yield of reaction, we deprotonated the starting material using sodium ethoxide generated *in situ* with ethanol. However, this resulted in a small quantity of the desired material and a large presence of side products which were difficult to separate hence use of Lu's method at 150°C was re-employed for the synthesis of key

intermediates **3** and **7**. As observed with substituted guanidines, the outcome of the aldol condensation reaction was observed to be sensitive to the hygroscopic nature of the supported potassium fluoride. The yield of the reaction was improved by recrystallisation of the aldehyde starting material and critically, intensive drying of KF-Alumina, without which the robustness of the reaction was compromised forming an unknown substance and lower yield of the desired material. By means of these modifications the desired product was produced in good yields by the aldol condensation of the appropriate hydroxybenzaldehyde and 2,7-dichlorofluorene with alumina supported potassium fluoride.

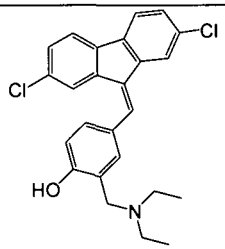
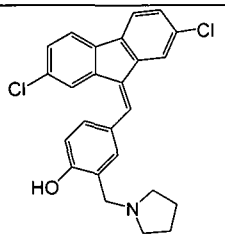
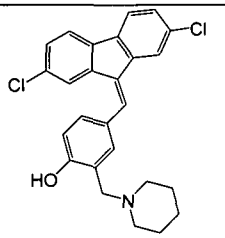
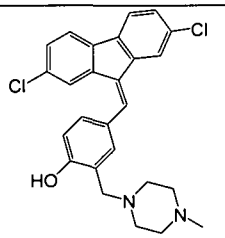
3.21.3 The Mannich Reaction

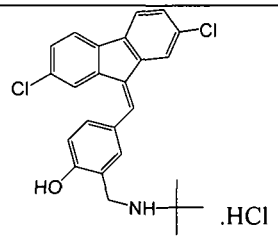
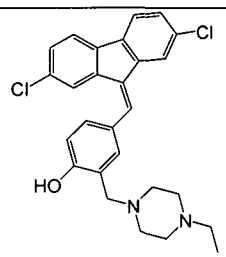
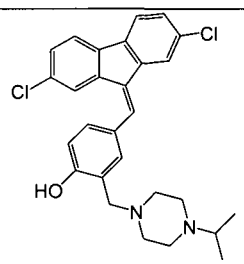
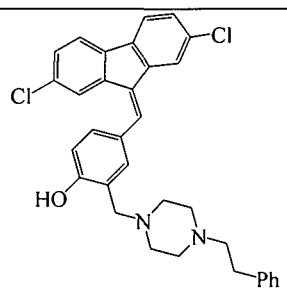
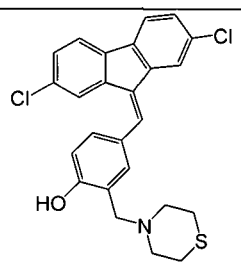
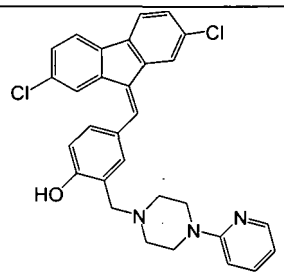
The Mannich reaction is recognised as being highly important for access to functionalised amines,[767,768] the catalytic asymmetric Mannich reaction being one of the most powerful methods for the construction of chiral nitrogen containing molecules.[769] Since the elucidation of the reaction mechanism in 1912 by Carl Mannich, the reaction has been modified to be controlled enantioselectively, stereoselectively and asymmetrically for the preparation of these organic building blocks.[769-776] The reaction was previously employed in our group for studies into the chemical basis of ADQ induced toxicity[777] by the introduction of a second dialkylamino group, and also for the development of less toxic analogues.[63,778,779] Typically the reaction takes place in the presence of formaldehyde and a primary or secondary amine usually in protic solvents. Examples of processes that utilize the Mannich reaction are varied, since many classes of biologically active molecules contain functionalities of this type including Tramadol® (analgesic), Moban® (management of schizophrenia) and Falicain® (local anesthetic) to name a few.

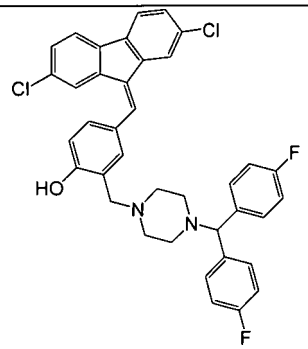
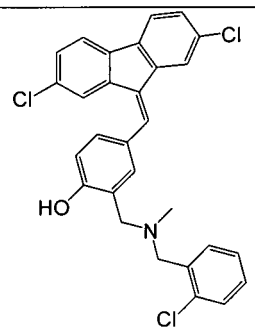
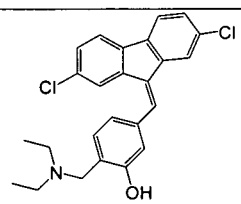
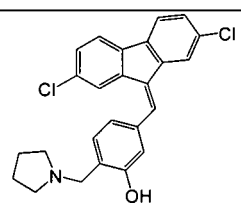
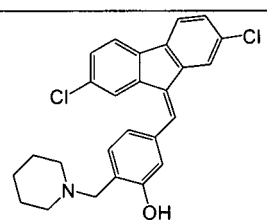
Hydroxy ylidene intermediates **111** and **124** were converted to their Fluorene-Mannich analogues **112-123** and **125-128** with formaldehyde and the appropriate amines under standard Mannich conditions to form the *mono* aminated products in good yields isolated by column chromatography (Table 15). Conversely the *meta* and *para* *t*-butylamine Mannich products **116** and **128** were found to ring close on standing confirmed by a characteristic peak in the ^{1}H NMR at 5.1 ppm and therefore were isolated as hydrochloride salts after treatment of the crude material with 20 % hydrochloric acid in *iso*-propyl alcohol. Other amines including 5-amino-2-

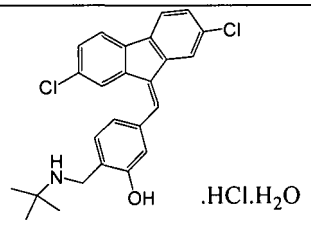
(trifluoromethyl)benzimidazole, 4-morpholinopiperidine, 1-(2-cyanophenyl)piperazine, N-(2-aminoethyl)morpholine, and 4-(1-pyrrolidinyl)piperidine were tried, however in the case of 4-morpholinopiperidine and 5-amino-2-(trifluoromethyl)benzimidazole, even with extended reaction times and addition of catalytic quantities of acetic acid and additional formaldehyde, the reaction was unsuccessful producing mainly starting material and an unidentified bi-product.

Table 15. Synthesis of Fluorene-Mannich Hybrids (with Lipinski's parameters)

Comp No.	Structure	Yield (%)	Formula weight	H bond acceptors	H bond donors	*Log P
112		41	$C_{25}H_{23}Cl_2NO$ 424.36	2	1	6.59
113		62	$C_{25}H_{21}Cl_2NO$ 422.35	2	1	6.23
114		67	$C_{26}H_{23}Cl_2NO$ 435.12	2	1	6.65
115		64	$C_{26}H_{24}Cl_2N_2O$ 451.39	3	1	5.67

116		33	$C_{25}H_{24}Cl_3NO$ 460.82	2	2	6.41
117		58	$C_{27}H_{26}Cl_2N_2O$ 465.41	3	1	6.01
118		55	$C_{28}H_{28}Cl_2N_2O$ 479.44	3	1	6.33
119		54	$C_{33}H_{30}Cl_2N_2O$ 541.51	3	1	7.68
120		54	$C_{25}H_{21}Cl_2NOS$ 454.41	2	1	6.41
121		48	$C_{30}H_{25}Cl_2N_3O$ 514.45	4	1	6.01

122		46	$C_{38}H_{30}Cl_2F_2N_2O$ 639.56	3	1	6.33
123		53	$C_{29}H_{22}Cl_3NO$ 506.85	2	1	8.20
125		57	$C_{25}H_{23}Cl_2NO$ 424.36	2	1	6.59
126		73	$C_{25}H_{21}Cl_2NO$ 422.35	2	1	6.23
127		72	$C_{26}H_{23}Cl_2NO$ 436.37	2	1	6.65

128		62	$C_{25}H_{26}Cl_3NO$ 478.84	2	2	6.41

*Log P calculated using ChemBioOffice 2008 predictive software

3.22 Fluorene Mannich Hybrids: Antiplasmodial Activity and Molecular Modelling

3.22.1 *In-Vitro* Parasitic Sensitivity

The Fluorene-Mannich (F-M) hybrids described here are designed based on the structural backbone of Lu. Explicitly the dichlorofluorene unit with substitution at C9, in addition, the nature of the Mannich side chain was chosen using Lipinski's rule of five as a reference for H-bonding and donating properties. Additionally the key pharmacophore features of CQ that are essential for activity were integrated. Molecular features were adapted in order to probe SAR. The concentration of drug required to inhibit parasitic growth by 50% (IC_{50}) as described previously was obtained using *P.falciparum* from various isolates is listed in Table 16. All hybrids were not equally potent but most did show activity in the nM range, some of which were greater than Lu (Table 17). The importance of the position and nature of the side chain is discussed and for completion, the SAR that the benzyl hydroxy group and carbon at position 9 impart. The latter two variants were synthesised by Dr Eleanor Row (University *of* Liverpool) and are included for the purpose of comparison.

Table 16. Origin and CQ sensitivity of lab strains of *P.falciparum* used in this chapter

Isolate	CQ Sensitivity	Origin
T994	Partial	Thailand
BC11	Resistant	Thai-Myanmar border in 2000
PCM6	Partial	Thai-Myanmar border in 2000
3D7	Sensitive	Unknown

Table 17. *In vitro* antimalarial activity of fluorene-ylidene derivatives

Comp number	*IC_{50} (nM) T994	*IC_{50} (nM) BC11	*IC_{50} (nM) PCM6
112	406.6 ± 151.9	303.0 ± 66.1	992.5 ± 151.1
113	172.5 ± 52.2	179.5 ± 50.2	660.1 ± 141.2
114	491.7 ± 115.9	273.8 ± 32.1	>1000
115	>1000	>1000	>2000
125	169.6 ± 48.6	198.6 ± 60.3	637.7 ± 151.4
126	36.7 ± 13.6	41.2 ± 11.2	150.9 ± 23.0
127	98.9 ± 18.9	70.3 ± 27.2	22.3 ± 41.8
Lu[1]	120.4 ± 37.3	89.0 ± 21.6	236.8 ± 74.6
CQ[2]	15.6 ± 4.8	123.7 ± 28.2	22.0 ± 8.0
MQ[3]	12.1 ± 2.2	10.1 ± 4.5	13.6 ± 5.0
Quin[4]	148.0 ± 36.2	278.3 ± 37.5	165.1 ± 24.2

[1]Lumefantrine; [2]Chloroquine; [3]Mefloquine; [4]Quinine *IC_{50} from an average of duplicate determinations

Table 18. *In vitro* antimalarial activity of fluorene-ylidene derivatives

Compound number	***IC_{50} (nM) 3D7**
113	71.9 ± 16.7
114	194.4 ± 19.1
115	281.9 ± 44.0
116	165.5 ± 104.7
117	>1000
118	>1000
119	>1000
120	>1000
121	>1000
122	>1000
123	>1000
126	22.3 ± 5.7
127	12.7 ± 5.3
128	40.2 ± 21.9
[1]Lu	40.3 ± 3.3
[2]CQ	4.5 ± 2.5

[1]Lumefantrine; [2]Chloroquine *IC_{50} calculated from an average of duplicate determinations

3.22.2 Size of the amine side chain and H-bond accepting capability.

The size of the side chain exerts an effect on the antiplasmodial activity as shown by Tables 17 and 18. As the size and degree of conjugation of the side chain increase, antimalarial activity decreases as displayed by compounds **119**, **121**, **122**, and **123**. The evidence is however inconclusive since the smaller side chains of compounds **117, 118,** and **120** are also poorly active. Also noted is the effect of replacing the terminal carbon of compound **114** for sulphur (compound **120**) which resulted in a decrease from 194 nM to >1000 nM.

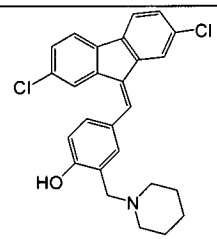
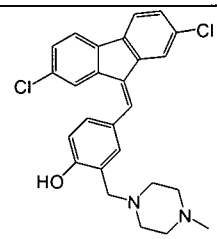
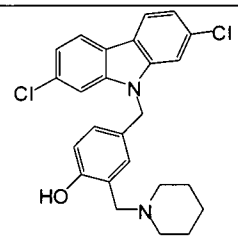
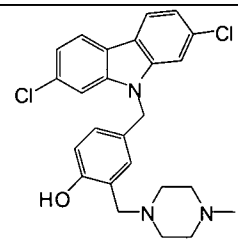
3.22.3 The importance of the hydroxyl group for activity; possible docking of OH to heme.

Dehydroxy versions of **112**, **125**, and **115** were synthesised in our laboratory by Dr Eleanor Row. For the methyl piperazine analogue **115**, this resulted in a decrease in activity from >1000 to >4000 nM in Thai isolates and 149 to >1000 nM in 3D7. The diethyl amine compounds **112** and **125** were tested *versus* their de-hydroxy analogue confirming the results obtained for the methyl piperazine analogue **115**. In the absence of the OH group, there is a marked decrease in activity from between 160 and 640 nM to >4000 nM. If the F-M hybrids do indeed bind to FPIX then this decrease in activity would be expected since the hydroxyl group is ideal for binding to the carboxylate residues of heme.

3.22.4 The effect of substitution at C9; *N*-alkyl carbazole compounds

N-Alkyl carbazole derivatives of compounds **114** and **115** were synthesised by Dr Eleanor Row and are included here for a comprehensive picture of structure activity relationships as illustrated in Table 19. These compounds were tested using CQ sensitive parasites. The lead compound of this series is the *N*-alkyl carbazole piperazine analogue **129** which is active in the 89 nM region compared to 194 nM for its benzylidiene analogue **114**. The activities of the carbazole series as a whole were poor. Nonetheless, when comparing these particular analogues **129** and **130** to the parent compounds **114** and **115**, the activity of the carbazoles is enhanced in orders of the same magnitude.

Table 19. Activity of *N*-alkyl carbazole analogues expressed as IC_{50}

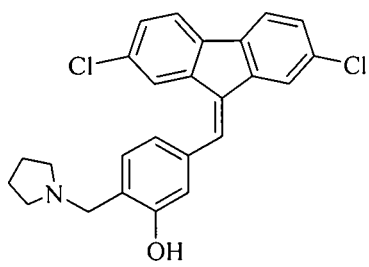
Compound Number	Structure	*IC_{50} (nM) 3D7
114		194.7 ± 27.0
115		281.9 ± 33.7
129		89.2 ± 22.7
130		170.8 ± 29.1

*Calculated from an average of triplicate determinations

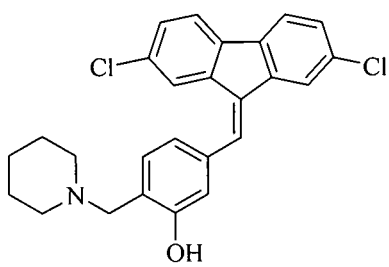
3.22.5 Structure activity relationships; nature of the amine side chain.

Of the variables tested, the nature of the Mannich side chain had the greatest effect. Seven compounds, **117-123** displayed poor activity against all strains of *P. falciparum* tested. Compounds **114** and **115** are more active against the CQ sensitive strains than those isolated from the Thai border with activities in the 100 and 1000 nM region respectively. It is clear that

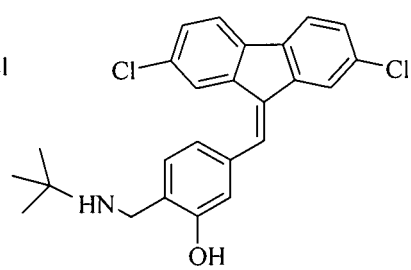
across the Thai isolates compound **115** is inactive, nonetheless against the CQ sensitive strain an improvement in activity is observed. Compounds **112**, **125**, **113** and **114** showed the most varying activity against the Thai isolates, with lowest activity observed against PCM6. Specifically, compounds **112** and **114** were most active against the BC11 strain whereas compounds **125** and **126** were most active against the T994 strain. Compound **115** was inactive *versus* the Thai isolates with IC_{50} between 1000 and 2000 nM decreasing to 281 nM when tested in the CQ sensitive system.



126



127



128

Figure 33. Compounds showing activity ≥ Lu

Three compounds (**126**, **127** and **128**) showed activity better than or comparable to Lu against all strains tested. The *t*-butyl amine derivative **128** showed activity in the range of Lu when tested against 3D7. *Meta* substituted analogues **126** and **127** showed the best activity. Lu had an IC_{50} of 40.3 nM in 3D7; compounds **126** and **127** are active in the 22.3 and 12.7 nM range respectively. For the Thai isolates, **126** and **127** again exhibit activity superior to that of Lu. Lu has inhibition concentrations of 120.4, 89.0 and 236.8 nM against the Thai isolates in the order T994, BC11, and PCM6. The piperidine analogue **127** has IC_{50} values of 98.9, 70.3, and 22.3 nM compared to those of compound **126**, 36.7, 41.2 and 150.9 nM. Compound **127** is more active than compound **126** against most of the strains tested and both compounds **126** and **127** are more active than Lu across all strains. In the BC11 strain CQ gave an activity of 123.7 nM, compounds **126** and **127** gave 41.2 and 70.3 nM respectively. When compared to the activity of quinine *versus* the Thai isolates, compounds **126** and **127** did better across the range the activity of compound **126** being unsurpassed.

The data set illustrates that for most analogues the optimal positions for antimalarial activity are for the Mannich side chain to be positioned *para* to the fluorene-benzylidene function and the

hydroxyl group *meta*. This is clearly illustrated by compounds **112** and **125**. When the hydroxyl group is *meta* the IC_{50} value is 169.6 nM, exchange of the hydroxyl and Mannich side chain positions leads to an inhibitory concentration of 406.6 nM. Comparison of compound **113** to its isomer **126** further supports this. In CQ sensitive isolates, if the side chain is in the *meta* position an IC_{50} of 71.9 nM is obtained, increasing to 22.3 nM when in the *para* position. This trend is also displayed with lead compound **127** compared to **114** and the *tert*-butyl amine derivatives **116** and **128**.

3.23 Inhibition of Hemozoin formation

The drug concentration required to inhibit formation of β-hematin by 50% (IC_{50}) was determined for each compound by Silvia Parapini (Università *di* Milano). The results are the mean IC_{50} of two different experiments performed in duplicate using the method of Parapini *et al.*[692] As previously mentioned, one of the biomolecular targets of antimalarial drug design is the process by which *P. falciparum* detoxifies monomeric heme released during haemoglobin catabolism to hemozoin. β-hematin is synthetic hemozoin identical in structure to plasmodial hemozoin. β-hematin can therefore be used as a seed for the formation of hemozoin *in vitro*, inhibition of which can be measured in order to ascertain the ability of a compound to inhibit the heme detoxification process.

Table 20. Inhibition of hemozoin formation *in vitro*

Compound	*F-P crystal growth Inhibition / μM
126	1.24
127	4.44
CQ	1.78

*Obtained from an average of triplicate determinations

Table 20 illustrates the ability of lead compounds **126** and **127** to inhibit the formation of hemozoin *in vitro* using CQ as a control. In our studies we found that CQ inhibits 50 % hemozoin formation at a concentration of 1.78 μM. When comparing this to the values obtained for compounds **126** and **127** it appears that compound **126** has the ability to inhibit the formation

of hemozoin at a concentration lower than that of CQ whereas compound **127** does inhibit but not within the range of CQ showing that Fluorene-Mannich hybrids have the capability of preventing hemozoin formation *in vitro* by probable binding to FPIX.

3.24 Molecular Modelling

In order to further investigate the potential interactions between the Fluorene-Mannich hybrid **126** and heme, molecular modelling was performed by Dr Neil Berry (University *of* Liverpool) using the Monte-Carlo method by means of the MMFF94 force field as illustrated in Figure 34. The low energy conformation generated for compound **126** reveals an interface between heme and **126** in the form of a π-π stacking interaction such that compound **126** could lay in the mode proposed here. In addition the terminal amine and hydroxyl functionalities are able to form a hydrogen bonding contact with the carboxylate residues of heme enhanced by a repulsive interaction between the fluorene hydrogen molecules and the phenyl ring causing this portion of the structure to twist in the manner depicted.

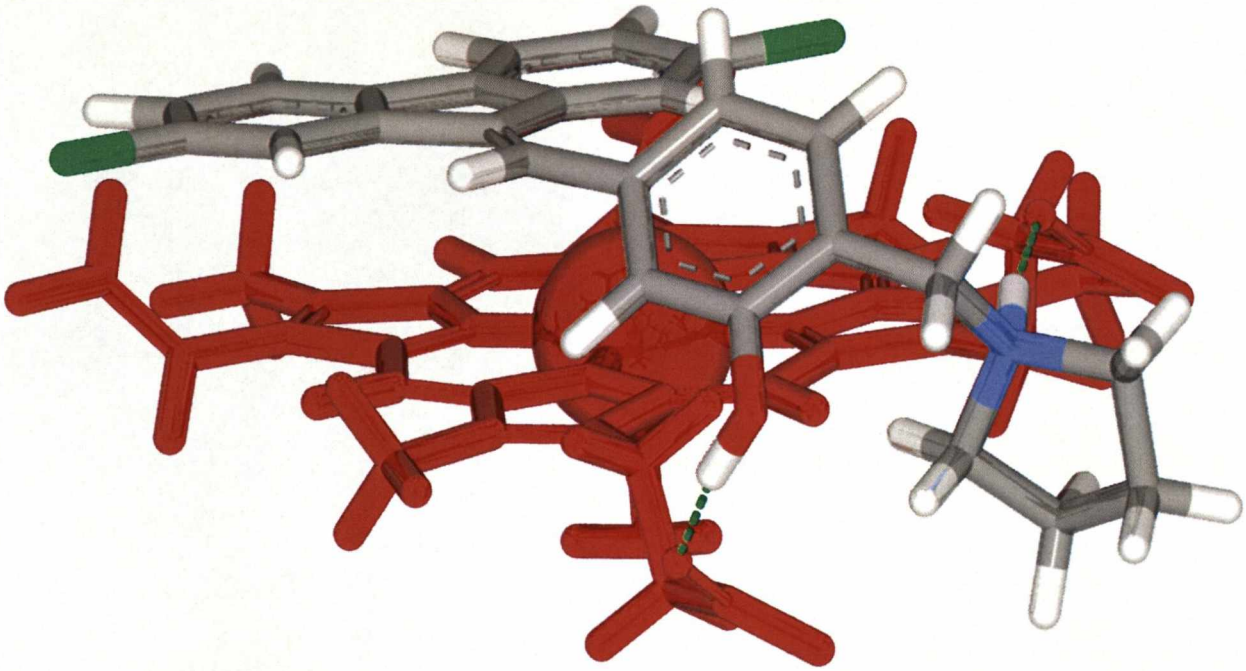


Figure 34. Molecular modelling image of the lowest energy conformation for the binding interaction of compound **126** with heme. Fluorene unit represented by; Cl = green, hydrogen = white, carbon = grey, blue = nitrogen, red = oxygen.

3.25 Conclusion and Future Work

We have successfully synthesised an array of F-M hybrids based on our knowledge of both the structural and chemical features of the fluorene template and our biomolecular target of interest, FPIX. The success of the synthetic route is dependent on the use of anhydrous KF-alumina in the aldol condensation step of the sequence furthermore the approach has major advantages in that it does not use costly reagents or starting materials. We have used fewer synthetic steps to construct *mono*-protic analogues that are superior in many cases to Lu. The results from anti-parasitic and molecular modelling data have inferred us to believe that the ability of a binding interaction between the hydroxyl group and carboxylates of heme is paramount for antimalarial activity of the F-M hybrids generated here as shown if Figure 35. We have demonstrated that these compounds are able to prevent the formation of β-hematin *in vitro*, adding weight to the

belief that they may be inhibitors of hemozoin formation *in vivo*, a known site of chemotherapeutic intervention for the cessation of parasitic development.

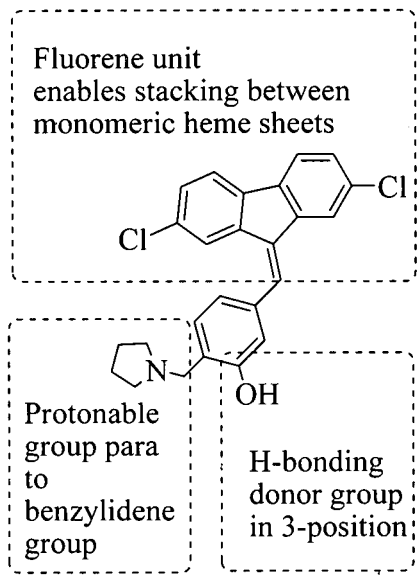


Figure 35. F-M hybrid pharmacophore

This is a preliminary investigation therefore further examination into their mechanism of action is required and *in vivo* activity data is being compiled at present (manuscript and patent application in preparation). The F-M template can be explored further especially within the carbazole series and we are currently outsourcing the synthesis of a range of analogues using microwave assisted synthesis in order to develop a more in-depth SAR profile.

CHAPTER IV

Conclusion

4.0 Conclusion

Beginning with the anti-trypanosomal drug pentamidine, we have successfully developed a novel antimalarial thiazole template for the treatment of uncomplicated malaria with potential use in combination chemotherapy. We have shown that this compound is able to act within the haemoglobin degradation pathway and therefore propose that this contributes to the mode of action. Further efforts will be directed towards establishing the significance of hemozoin inhibition in the mode of action, primarily by quantifying the effect inhibitors of haemoglobin digestion have on their activity. Here we show that thiazole diamidines are not recognised by the chloroquine resistance mechanism thus offering advantages over other hematin binding drugs. Furthermore since these drugs are impermeable to human erythrocytes they are selective for parasitised cells.

Compound **62** is a potent antimalarial with activity superior to DB75. Furthermore, cytotoxicity studies show that thiazole **62** is less toxic than DB75 and has a high therapeutic index. These factors indicate that this analogue may have excellent therapeutic potential and thus an evaluation of the oral activity of corresponding prodrugs is underway in the malaria animal model. In addition, the stability of the thiazole heterocycle may impart increased metabolic stability on this molecule.

The synthesis of thiazole **62** is however compounded by cyanation and the Pinner mediated diamidine incorporation. Development of alternative methods to the formation of the *bis*-nitrile and *bis*-amidine will be investigated within the drug optimisation stage. With respect to thiazole prodrugs, synthetic routes to the formation of sulfonyl derivatives will also be addressed.

Considerable effort has been made toward the cyanation of diphenylisoxazoles however, issues regarding the formation of the *bis*-nitrile led to failure with this template. In contrast, fluorene diguanidine compounds were successfully synthesised using a route enabling access to the prodrug. Unfortunately, the parent compounds are poorly active against CQ resistant parasites and moderately active against sensitive isolates. Nonetheless we feel that these compounds may have potential and therefore further work regarding their activity *versus* the K1 CQ resistant

strain will be carried out. Furthermore, issues surrounding the solubility of these drugs in the assay system may account for their low activity, thus the *in vivo* activity of Boc prodrugs will be investigated within the group.

A novel series of inhibitors of malaria pigment hemozoin have been designed based on the structural properties of chloroquine, amodiaquine and lumefantrine. Important structural features for activity include a flat heme binding template and protonable side chain. Inhibition of hemozoin formation is a proven target for the most effective antimalarial drugs. From our series, lead compounds with low nanomolar activity were identified and subsequent molecular modelling studies were performed. From initial modelling studies we have identified that the fluorene unit is capable of π-π stacking over the porphyrin heme ring system and that the amodiaquine like side chain can hydrogen bond with the propionate groups of hematin. Substitution of the aryl ring with a *meta*-hydroxyl function and a *p*-substituted Mannich side-chain is optimal for binding. The modelling is supported by β-hematin inhibition assays in the sense that these molecules have comparable IC_{50}s to existing antimalarials such as CQ and amodiaquine.

These compounds are potent against CQ sensitive parasites loosing activity against CQ resistant ones, exemplified by lead compound **127** with IC_{50}s of 12.7 and 70.3 nM respectively. However, with activity superior to lumefantrine, F-M hybrids have the potential to be utilised within combination therapy, therefore ongoing studies are focused on the *in vivo* activity of these compounds. Preliminarily studies suggest that lead compound **127** also possess potent activity *in vivo*.

CHAPTER V

Experimental Details

5.0 Experimental Details

5.1 General Experimental Details

5.1.1 Purification of Reagents

All solvents and chemicals were purchased from Sigma-Aldrich Chemical Company and used without purification aside from: *p*-hydroxybenzaldehyde and *m*-hydroxybenzaldehyde which were purified from $CHCl_3$ and dried under vacuum overnight, NCS which was recrystallisation from water and dried in the same manner.

5.1.2 Purification of Solvents

Diethyl ether and THF were freshly distilled under a constant flow of nitrogen, from the sodium / benzophenone ketyl radical, prior to use. Dichloromethane was stirred over calcium hydride for 12 hours prior to distillation under dry nitrogen.

5.1.3 Preparation of Glassware

All syringes, flasks, needles, condensers and stirrer bars were dried in an oven overnight at 150-200°C prior to use. Flasks, condensers and stirrer bars were cooled under a positive nitrogen pressure prior to use. All anhydrous reaction were performed with a small, static pressure of nitrogen.

5.1.4 Chromatography

Thin-layer chromatography (TLC) was carried out on aluminium-backed Merck Kiesgel plates (Merck, Darmstadt, Germany) with detection by UV (254 nm) fluorescence. Flash column chromatography was carried out using Merck Silica gel 60 (<63 μm) or Fisher Matrex 35 to 70 μm eluting with various solvent mixtures and using bellows to apply pressure.

5.1.5 Spectroscopy

NMR spectra were recorded employing a Bruker AMX 400 (^{1}H, 400 MHz; ^{13}C, 100 MHz) spectrometer. Chemical shifts (δ) are reported in parts per million downfield relative to TMS as internal standard. Coupling constants (*J*) are in Hertz (Hz). Chemical shifts were referenced to residual non deuterated solvent present in the deuterated sample, e.g., $CHCl_3$ in $CDCl_3$. The following symbols have been adopted for the description of NMR spectra:

s = singlet
d = doublet
dd = doublet of doublets
t = triplet
q = quartet
m = multiplet
br = broad

Chemical ionization (CI), electron impact (EI) and electro spray (ESP) mass spectrometry were recorded on VG analytical 7070E and Fisons TRIO machines by the University *of* Liverpool Mass Spectrometry Department. In the description of mass spectra M^+ corresponds to the molecular ion peak.

Elemental analysis was performed by the University *of* Liverpool Microanalysis laboratory.

Infra-red spectra were recorded in the range 4000-600 cm^{-1} using a Perkin-Elmer 1320 where samples were run as nujol / liquid paraffin mulls on sodium chloride plates and a JASCO FT/IR-4100 spectrophotometer were samples were obtained neat.

5.1.6 Other Data

Molecular Modelling

For PMD analogues (Section 3.1) a conformational search using a Monte-Carlo method using the MMFF94 forcefield was performed on the diprotonated molecules.[i] The Boltzmann weighted average of the inter amidine separation (as measured from the carbon bonded to the two nitrogens) was calculated for each molecule. These values were plotted against $\log_{10}$(-Activity) using the TM6 and HB3 data.

In order to generate a model for the binding of molecules **7**, **61** and **126** with heme a conformational search using a Monte-Carlo method with the MMFF94 forcefield was performed on the chosen compound with a molecule of heme.

[i]Spartan'04, Wavefunction, Inc., Irvine, CA. http://www.wavefun.com/

Melting points were determined on a Stuart SMP3 and Gallenkamp melting point apparatus quoted uncorrected in degrees Centigrade.

5.1.7 Analytical Reverse-Phase HPLC

Reverse-phase HPLC was performed on a 250 mm x 4.6 mm, Gemini 5u C18 110 Å reverse-phase column supplied from Phenomonex® eluting with a two buffer system; Buffer A: 0.1 M aqueous TEAB solution; Buffer B: 0.1 M aqueous TEAB solution containing 40% acetonitrile.

5.1.8 Preparative HPLC

Preparative HPLC was also performed using a 250 x 20 mm I.D, 5 μM YMC-pack ODS-A column supplied from YMC Europe GmbH eluting with MeCN: Water 0.1% TFA (20-80 % gradient over 20 min). After removal of solvent the compounds were isolated as their respective TFA salts.[706] Columns were stored in HPLC grade MeCN.

5.1.9 Preparation of HPLC Reagents

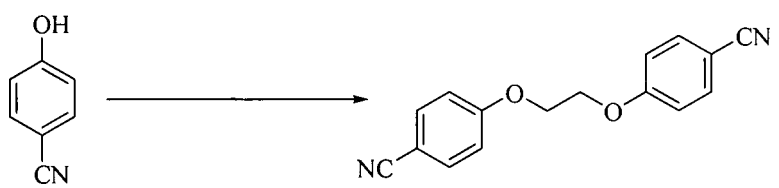
1 M aqueous TEAB solution: prepared by bubbling carbon dioxide gas through a 1 M aqueous triethylamine solution until attaining *p*H 7.65. Buffer A (0.1 M aqueous TEAB solution) was prepared by diluting 100 mL of the 1 M aqueous TEAB solution with 900 mL of distilled water to make 1 L of buffer A. Buffer B (0.1 M aqueous TEAB solution containing 40 % acetonitrile) was prepared by diluting 100 mL of the 1 M aqueous TEAB solution with 400 mL of HPLC grade acetonitrile and 500 mL of distilled water to make 1 L of buffer B.

Water containing 0.1 % TFA was prepared by addition of 1 g TFA to 1 L of HPLC grade water.

5.1.10 Other Apparatus

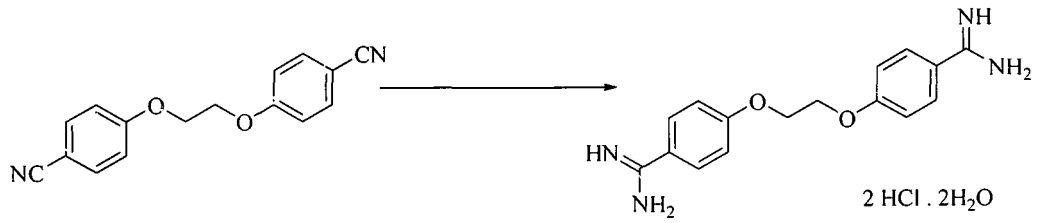
HCl gas was generated *via* a steel cylinder purchased from Boc gases. H_2S gas was generated *via* a 227 g steel cylinder purchased from Sigma-Aldrich Chemical Company.

5.2 Individual Procedures

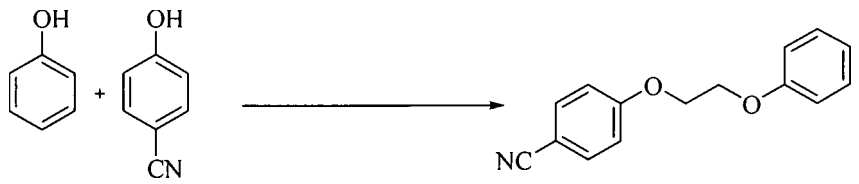


1,2-Bis (4-cyanophenoxy) ethane (2) Sodium (0.16 g, 6.96 mmol) was added portionwise to anhyd. EtOH (4.0 mL) under an atmosphere of nitrogen. After dissolution of Na, a solution of 4-cyanophenol (0.75 g, 6.38 mmol) dissolved in anhyd. EtOH (4.0 mL) was added followed by dropwise addition of 1,2-dibromoethane (0.28 mL, 3.19 mmol). The reaction mixture was stirred at reflux under a nitrogen atmosphere for 3 days after which the mixture was cooled, filtered, the solid washed with water and dried under vacuum. Purification by column chromatography eluting with DCM: hexane (8:2) gave the desired compound **2** as a white solid (1.42g, 84%). Mp

211-212°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.61 (d, 4H, J = 9.0 Hz, ArH), 7.01 (d, 4H, J = 9.0 Hz, ArH), 4.39 (s, 4H, CH_2); ^{13}C NMR ($CDCl_3$, 100MHz) δ 161.6, 134.1, 118.9, 115.3, 104.7, 66.4; ν_{max} (Nujol) /cm^{-1} 3326 (C-O-C), 3033 (ArH), 2898 (C-H), 2223 (C≡N), 1602(Ar), 1509(Ar), 1247 (C-O-C); *m/z* (CI) 282 ([M+NH_4]$^+$), found 282.12433, $C_{16}H_{16}O_2N_3$ requires 282.12424; anal. Found C 72.07, H 4.51, N 10.54, $C_{16}H_{12}O_2N_2$ requires C 72.71, H 4.57, N 10.60.



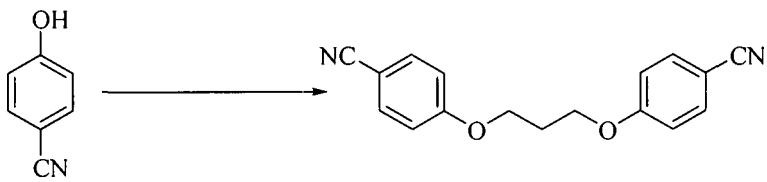
4,4'-(ethane-1,2-diyl*bis*(oxy))dibenzimidamide dihydrochloride dihydrate (6) Compound **2** (0.51 g, 1.92 mmol) was dissolved in a mixture of anhyd. benzene (54 mL) and EtOH (2.90 mL), cooled to 0 ^{0}C and saturated with HCl gas. The mixture was sealed and stirred at room temperature for 3 days after which anhyd. Et_2O (28 mL) was introduced and the mixture stirred for 10 minutes. The solids were filtered under nitrogen and dissolved in a mixture of anhydrous EtOH (36 mL) and EtOH.NH_3 (36 mL). The mixture was heated overnight (50 ^{0}C), cooled to room temperature and reduced by half *in vacuo*. Ether was added to precipitate the solid which was filtered, washed and dried under vacuum. Purification by recrystallisation (2N HCl) gave the desired compound **6** as fine white needles (0.54 g, 70%). Mp 333°C; ^{1}H NMR (MeOD, 400 MHz) δ 7.85 (d, 4H, J = 9.0 Hz, ArH), 7.23 (d, 4H, J = 9.0 Hz, ArH), 4.52 (s, 4H, CH_2); ^{13}C NMR (MeOD, 100MHz) δ 167.9, 165.3, 131.5, 121.7, 116.7, 68.5; ν_{max} (Nujol) / cm^{-1} 3362 (N-H), 3037 (ArH), 2940 (C-H), 1658 (C=N-H), 1606 (Ar), 1505 (Ar), 1245(C-O-C); *m/z* (ESP) 299 ([M-H]$^-$); anal. Found C 47.54 H 5.62 N 14.30, $C_{16}H_{24}Cl_2N_4O_4$ requires C 47.18, H 5.94, N 13.76.



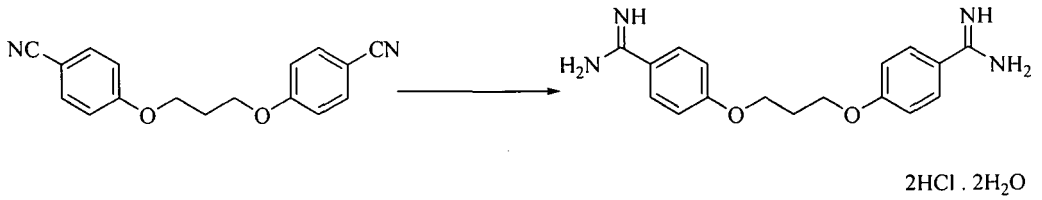
4-(2-phenoxyethoxy)benzonitrile (10) Sodium (0.15 g, 6.52 mmol) was added portionwise to dry EtOH (4.0 mL) under an atmosphere of nitrogen. After dissolution of Na, a solution of 4-cyanophenol (0.50 g, 4.19 mmol) dissolved in dry ethanol (4.0 mL) was added followed by dropwise addition of 1,2 dibromoethane (0.36 mL, 4.19 mmol). The reaction mixture was stirred at reflux and monitored by TLC. After consumption of 4-cyanophenol the reaction mixture was cooled to room temperature. In a separate flask Na (0.15 g, 6.52 mmol) was added portionwise to dry EtOH (4.0 mL) and dissolved under a nitrogen atmosphere. A solution of phenol (0.39 g, 4.19 mmol) in ethanol (4.0 mL) was added and stirred for 10 minutes. This was added dropwise to the cooled mixture and stirred at reflux for 3 days after which the mixture was cooled, filtered, the solid washed with water and dried under vacuum. Purification by column chromatography eluting with DCM: hexane (8:2) gave the desired compound **10** as a white solid (0.72 g, 72%). Mp 134-135°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.60 (d, 2H, J = 9.0 Hz, ArH), 7.31 (m, 2H, ArH), 6.97 (m, 5H, ArH), 4.35 (m, 4H, CH_2); ^{13}C NMR ($CDCl_3$, 100MHz) δ 162.3, 158.7, 139.2, 134.4, 129.9, 121.7, 119.5, 115.7, 115.0, 67.2, 66.4; ν_{max} (Nujol) / cm^{-1} 3297 (N-H), 2929 (C-H), 2223(C≡N), 1602 (Ar), 1504 (Ar), 1236 (C-O-C); *m/z* (CI) 257 ([M+NH_4]$^+$), found 257.12881, $C_{15}H_{17}N_2O_2$ requires 257.12900; anal. Found C 75.20, H 5.49, N 5.81, $C_{15}H_{13}NO_2$ requires C 75.30, H 5.48, N 5.85.

4-(2-Phenoxyethoxy)benzamidine hydrochloride hydrate (12) Compound **10** (0.27 g, 1.06 mmol) was dissolved in a mixture of anhyd. benzene (100 mL) and ethanol (1.56 mL), cooled to 0 ^{0}C and saturated with HCl gas. The mixture was sealed and stirred at room temperature for 3 days after which anhyd. Et_2O (16 mL) was introduced and the mixture stirred for 10 minutes.

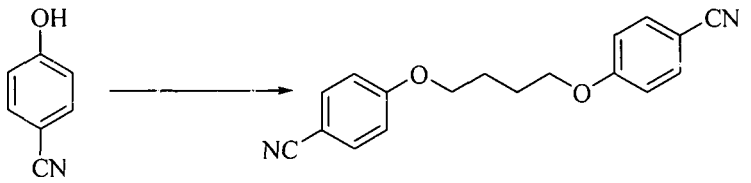
The solids were filtered under nitrogen and dissolved in a mixture of anhyd. EtOH (20 mL) and anhyd. EtOH.NH_3 (20 mL). The mixture was heated overnight (50 ^{0}C), cooled to room temperature and reduced by half *in vacuo*. Ether (30 mL) was added to precipitate the solid which was filtered, washed and dried under vacuum. Purification by recrystallisation (2N HCl) gave the desired compound **12** as fine white needles (0.24 g, 73%). Mp 209°C; ^{1}H NMR (MeOD, 400 MHz) δ 7.81 (d, 2H, J = 9.0 Hz, ArH), 7.28 (m, 2H, ArH), 7.20 (d, 2H, J = 9.0 Hz, ArH), 6.94 (d, 3H, J = 8.0 Hz, ArH), 4.45 (m, 2H, CH_2), 4.36 (m, 2H, CH_2); ^{13}C NMR (MeOD, 100MHz) δ 167.9, 165.3, 164.8, 131.4, 130.9, 122.5, 116.8, 116.1, 68.9, 68.0; ν_{max} (Nujol) / cm^{-1} 3311 (N-H), 3143 (Ar-H), 2929 (C-H), 1650 (C=N), 1602 (Ar), 1504 (Ar), 1240 (C-O); *m/z* (ESP) 257.1 ([M+H]$^+$), found 257.1279, $C_{15}H_{17}N_2O_2$ requires 257.1290; anal. Found C 58.50 H 6.20 N 9.09, $C_{15}H_{19}ClN_2O_3$ requires C 57.97, H 6.16, N 9.01.



4, 4'-(propane-1,3-diyl*bis*(oxy))dibenzonitrile (3) Sodium (0.16 g, 6.96 mmol) was added portionwise to anhyd. EtOH (4.0 mL) stirring under an atmosphere of nitrogen. After dissolution of Na, a solution of 4-cyanophenol (0.75 g, 6.38 mmol) dissolved in dry ethanol (4.0 mL) was added followed by dropwise addition of 1,3-dibromopropane (0.32 mL, 3.19 mmol). The reaction mixture was stirred at reflux under a nitrogen atmosphere for 3 days after which the mixture was cooled, filtered, the solid washed with water and dried under vacuum. Purification by column chromatography eluting with DCM: hexane (8:2) gave the desired compound **3** as a white solid (1.40 g, 79%). Mp 190-191°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.59 (d, 4H, J = 8.5 Hz, ArH), 6.96 (d, 4H, J = 8.5 Hz, ArH), 4.20 (t, 4H, J = 6.0 Hz, CH_2), 2.32 (m, 2H, CH_2); ^{13}C NMR ($CDCl_3$, 100MHz) δ 161.9, 134.0, 119.1, 115.1, 104.2, 64.4, 28.8; ν_{max} (Nujol) / cm^{-1} 3104 (Ar-H), 2823 (C-H), 2221 (C≡N), 1604 (Ar), 1509 (Ar), 1253 (C-O); *m/z* (CI) 296 ([M+NH_4]$^+$), found 296.14037, $C_{17}H_{18}N_3O_2$ requires 296.13992; anal. Found C 73.22, H 5.13, N 10.03, $C_{17}H_{14}N_2O_2$ requires C 73.37, H 5.07, N 10.07.

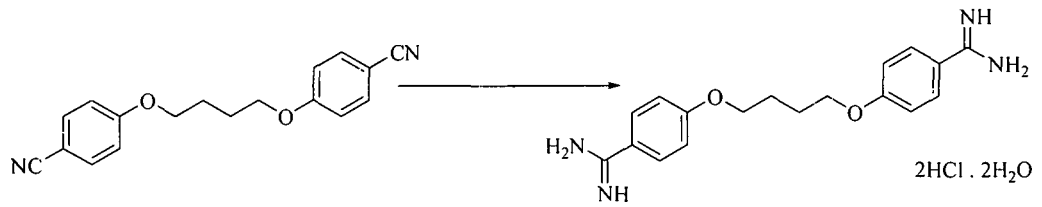


4,4'-(propane-1,3-diyl*bis*(oxy))dibenzimidamide dihydrochloride dihydrate (7) Compound **3** (0.50 g, 1.79 mmol) was dissolved in a mixture of anhyd. benzene (55 mL) and anhyd. ethanol (3.0 mL), cooled to 0 ^{0}C and saturated with HCl gas. The mixture was sealed and stirred at room temperature for 3 days after which ether (30 mL) was introduced and the mixture stirred for 10 minutes. The solids were filtered under nitrogen and dissolved in a mixture of anhyd. EtOH (36 mL) and EtOH.NH_3 (36 mL). The mixture was heated overnight (50 ^{0}C), cooled to room temperature and reduced by half *in vacuo*. Et_2O (15 mL) was added to precipitate the solid which was filtered, washed and dried under vacuum. Purification by recrystallisation (2N HCl) gave the desired compound **7** as fine white needles (0.59 g, 78%). Mp 200°C ^{1}H NMR (MeOD, 400 MHz) δ), 7.82 (d, 4H, J = 9.0 Hz, ArH), 7.19 (d, 4H, J = 9.0 Hz, ArH), 4.34 (t, 4H, J = 6.0 Hz, CH_2), 2.46 (m, 2H, CH_2); ^{13}C NMR (MeOD, 100MHz) δ 167.9, 165.5, 131.5, 121.5, 121.4, 116.6, 66.4, 30.3; ν_{max} (Nujol) / cm^{-1} 3280 (N-H), 3038 (Ar-H), 2929 (C-H), 1504 (Ar), 1606 (Ar), 1240 (C-O-C); *m/z* (ESP) 313 ([M-H]$^-$); anal. Found C 48.60 H 6.10 N 13.25, $C_{17}H_{26}Cl_2N_4O_4Cl_2$ requires C 48.46, H 6.22, N 13.30.

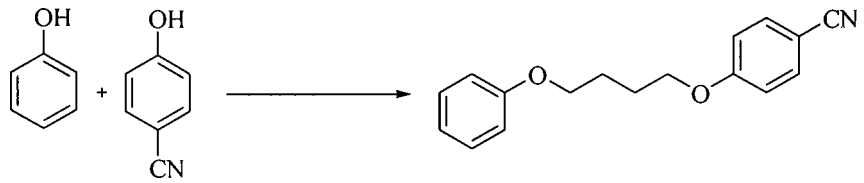


4,4'-(butane-1,4-diyl*bis*(oxy))dibenzonitrile (4) Sodium (0.10 g, 4.35 mmol) was added portionwise to dry EtOH (4.0 mL) stirring under an atmosphere of nitrogen. After dissolution of Na, a solution of 4-cyanophenol (0.47 g, 3.95 mmol) dissolved in dry ethanol (4.0 mL) was added followed by dropwise addition of 1,4-dibromobutane (0.24 mL, 1.98 mmol). The reaction mixture was stirred at reflux under a nitrogen atmosphere for 3 days after which the mixture was

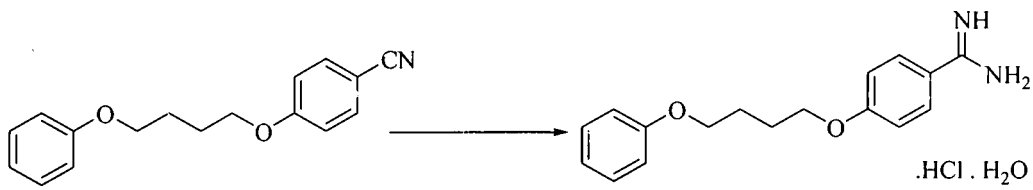
cooled, filtered, the solid washed with water and dried under vacuum. Purification by column chromatography eluting with DCM: hexane (8:2) gave the desired compound **4** as a white solid (1.03 g, 89%). Mp 174°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.59 (d, 4H, J = 8.9 Hz, ArH), 6.93 (d, 4H, J = 8.9 Hz, ArH), 4.08 (m, 4H, CH_2), 2.01 (m, 4H, CH_2); ^{13}C NMR ($CDCl_3$, 100MHz) δ 162.1, 134.0, 119.1, 115.1, 104.0, 67.7, 25.7; ν_{max} (Nujol) / cm^{-1} 3332 (C-O-C), 3033 (Ar-H), 2956 (C-H), 2219 (C≡N), 1604 (Ar), 1506 (Ar), 1251 (C-O-C); *m/z* (CI) 310 ([M+NH_4]$^+$), found 310.15532, $C_{18}H_{20}N_3O_2$ requires 310.15555; anal. Found C 74.03, H 5.55, N 9.55, $C_{18}H_{16}N_2O_2$ requires C 73.95, H 5.52, N 9.58.



4,4'-(butane-1,4-diyl*bis*(oxy))dibenzimidamide dihydrochloride dihydrate (8) Compound **4** (0.42 g, 1.44 mmol) was dissolved in a mixture of anhyd. benzene (46 mL) and anyhd. ethanol (2.50 mL), cooled to 0 ^{0}C and saturated with HCl gas. The mixture was sealed and stirred at room temperature for 3 days after which anyhd. Et_2O (40 mL) was introduced and the mixture stirred for 10 minutes. The solids were filtered under nitrogen and dissolved in a mixture of anhydrous EtOH (34 mL) and EtOH.NH_3 (34 mL). The mixture was heated overnight (50 ^{0}C), cooled to room temperature and reduced by half *in vacuo*. Ether (15 mL) was added to precipitate the solid which was filtered, washed and dried under vacuum. Purification by recrystallisation (2N HCl) gave the desired compound **8** as fine white needles (0.46 g, 73%). Mp 286-287°C; ^{1}H NMR (MeOD, 400 MHz) δ, 7.80 (d, 4H, J = 9.0 Hz, ArH), 7.14 (d, 4H, J = 9.0 Hz, ArH), 4.19 (m, 4H, CH_2), 2.02 (m, 4H, CH_2); ^{13}C NMR (MeOD, 100MHz) δ 165.7, 131.4, 121.2, 116.7, 69.7, 27.2; ν_{max} (Nujol) / cm^{-1} 3370 (N-H), 3129 (Ar-H), 2884 (C-H), 1650 (C=N), 1606 (Ar), 1508 (Ar), 1257 (C-O); *m/z* (ESP) 327 ([M+H]$^+$); anal. Found C 49.87 H 6.46 N 12.67, $C_{18}H_{28}Cl_2N_4O_4$ requires C 49.66, H 6.48, N 12.87.

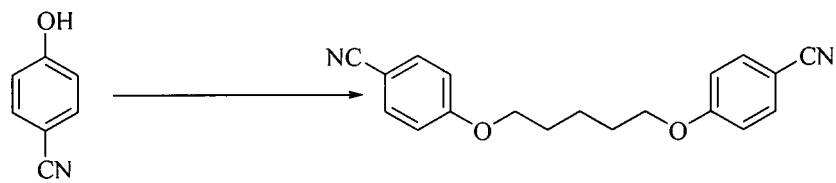


4-(4-Phenoxybutoxy)benzonitrile (11) Sodium (0.16 g, 6.96 mmol) was added portionwise to dry EtOH (5 mL) and dissolved under a nitrogen atmosphere. To this a solution of 4-cyanophenol (0.53 g, 4.47 mmol) dissolved in dry ethanol (5 mL) was added followed by dropwise addition of 1,4-dibromobutane (0.53 mL, 4.47 mmol). The reaction mixture was stirred at reflux and monitored by TLC. After consumption of 4-cyanophenol the reaction mixture was cooled to room temperature. In a separate flask Na (0.16 g, 6.96 mmol) was added portionwise to dry EtOH (5 mL) stirring under nitrogen. A solution of phenol (0.42 g, 4.47 mmol) in ethanol (5 mL) was added and stirred for 10 minutes. This mixture was added dropwise to the cooled mixture and stirred under reflux for 3 days after which the mixture was cooled, filtered, the solid washed with water and dried under vacuum. Purification by column chromatography eluting with DCM: hexane (8:2) gave the desired compound **11** as a white solid (0.98 g, 82%). Mp 130°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.57 (d, 2H, *J* = 8.9 Hz, ArH), 7.28 (d, 1H, *J* = 7.5 Hz, ArH), 7.26 (d, 1H, *J* = 8.1 Hz, ArH), 6.92 (m, 5H, ArH), 4.08 (t, 2H, *J* = 5.9Hz, CH_2), 4.03 (t, 2H, *J* = 5.9Hz, CH_2), 1.99 (m, 4H, CH_2); ^{13}C NMR ($CDCl_3$, 100MHz) δ 162.6, 159.2, 134.3, 129.8, 121.1, 115.5, 114.8, 104.3, 68.3, 67.5, 26.2; ν_{max} (Nujol) / cm^{-1} 3043 (Ar-H), 2884 (C-H), 2219 (C≡N), 1602 (Ar), 1504 (Ar), 1247 (C-O); *m/z* (CI) 285 ($[M+NH_4]^+$), found 285.16020, $C_{17}H_{21}N_2O_2$ requires 285.16031; anal. Found C 76.40, H 6.46, N 5.44, $C_{17}H_{17}NO_2$ requires C 76.38, H 6.40, N 5.24.

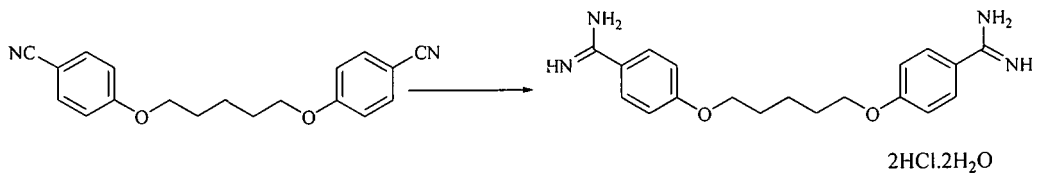


4-(4-Phenoxybutoxy)benzimidamide hydrochloride hydrate (13) Compound **11** (0.27 g, 1.01 mmol) was dissolved in a mixture of dry benzene (100 mL) and ethanol (1.60 mL), cooled to 0 ^{0}C and saturated with HCl gas. The mixture was sealed and stirred at room temperature for 3 days after which anhyd. Et_2O (16 mL) was introduced and the mixture stirred for 10 minutes. The solids were filtered under nitrogen and dissolved in a mixture of anhyd. EtOH (20 mL) and

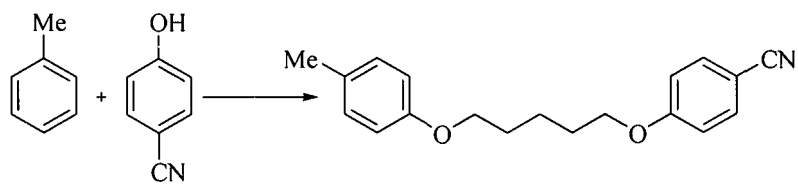
anhyd. EtOH.NH_3 (20 mL). The mixture was heated overnight (50 ^{0}C), cooled to room temperature and reduced by half *in vacuo*. Ether (30 mL) was added to precipitate the solid which was filtered, washed and dried under vacuum. Purification by recrystallisation (2N HCl) gave the desired compound **13** as fine white needles (0.28 g, 82%). Mp 134-135°C; ^{1}H NMR (DMSO, 400 MHz) δ 9.28 (s, 2H, NH_2), 9.08 (s, 2H, NH_2), 7.86 (d, 2H, J = 9.0 Hz, ArH), 7.29 (d, 1H, J = 7.0 Hz, ArH), 7.27 (d, 1H, J = 7.2 Hz, ArH), 7.16 (d, 2H, J = 9.0 Hz, ArH), 6.93 (d, 3H, J = 7.8 Hz, ArH), 4.16 (t, 2H, J = 5.9 Hz, CH_2), 4.03 (t, 2H, J = 5.9 Hz, CH_2), 1.89 (m, 4H, CH_2); ^{13}C NMR (DMSO, 100MHz) δ 165.0, 163.3, 158.9, 130.5, 129.8, 120.7, 119.6, 115.1, 114.7, 68.1, 67.2, 25.6, 25.5; ν_{max} (Nujol) / cm^{-1} 3288 (N-H), 1656 (C=N-H), 1604 (Ar), 1506 (Ar), 1234 (C-O-C); *m/z* (ESP) 285 ([M+H]$^+$), found 285.1603, $C_{17}H_{24}N_2O_2$ requires 285.1599; anal. Found C 59.50 H 6.75 N 8.33, $C_{17}H_{23}ClN_2O_3$ requires C 60.26, H 6.84, N 8.27.



4,4'-(pentane-1,5-diyl*bis*(oxy))dibenzonitrile (5) Sodium (0.11 g, 4.78 mmol) was added portionwise to anhyd. EtOH (5 mL) and dissolved under nitrogen atmosphere. To this a solution of 4-cyanophenol (0.52 g, 4.38 mmol) in anhyd. ethanol (5 mL) was added followed by dropwise addition of 1,5 dibromopentane (0.30 mL, 2.20 mmol). The reaction mixture was stirred at reflux under a nitrogen atmosphere for 3 days after which the mixture was cooled, filtered, the solid washed with water and dried under vacuum. Purification by column chromatography eluting with DCM: hexane (8:2) gave the desired compound **5** as a white solid (1.12 g, 83%). Mp 116-118°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.58 (d, 4H, J = 9.0 Hz, ArH), 6.93 (d, 4H, J = 9.0 Hz, ArH), 4.03 (t, 4H, J = 6.4, CH_2), 1.89 (m, 4H, CH_2), 1.68 (m, 2H, CH_2); ^{13}C NMR ($CDCl_3$, 100MHz) δ 162.6, 134.3, 119.6, 115.5, 104.2, 68.4, 31.3, 29.1, 23.0; ν_{max} (Nujol) / cm^{-1} 3095 (Ar-H), 2873 (C-H), 2221 (C≡N), 1604 (Ar), 1508 (Ar), 1259 (Ar); *m/z* (CI) 324 ([M+NH_4]$^+$), found 324.17142, $C_{19}H_{22}N_3O_2$ requires 324.17120; anal. Found C 73.99, H 5.98, N 8.94, $C_{19}H_{18}N_2O_2$ requires C 74.49, H 5.92, N 9.14.

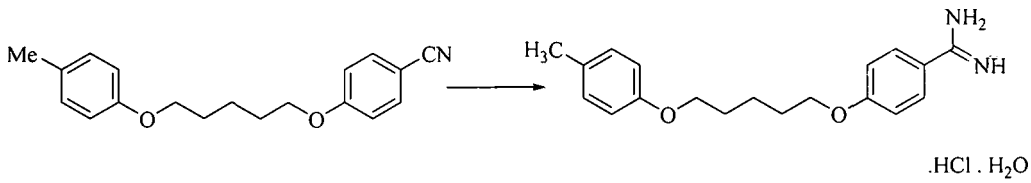


4,4'-(pentane-1,5-diyl*bis*(oxy))dibenzimidamide dihydrochloride dihydrate (9) Compound **5** (0.28 g, 0.91 mmol) was dissolved in a mixture of anhyd. benzene (107 mL) and ethanol (5.71 mL), cooled to 0 ^{0}C and saturated with HCl gas. The mixture was sealed and stirred at room temperature for 3 days after which anhyd. Et_2O (20 mL) was introduced and the mixture stirred for 10 minutes. The solids were filtered under nitrogen and dissolved in a mixture of anhydrous EtOH (74 mL) and $EtOH.NH_3$ (74 mL). The mixture was heated overnight (50 ^{0}C), cooled to room temperature and reduced by half *in vacuo*. Ether (30 mL) was added to precipitate the solid which was filtered, washed and dried under vacuum. Purification by recrystallisation (2N HCl) gave the desired compound **9** as fine white needles (0.33 g, 81%). Mp 243-245°C; ^{1}H NMR (DMSO, 400 MHz) δ, 9.31 (s, 4H, NH_2), 9.09 (s, 4H, NH_2), 7.88 (d, 4H, J = 8.9 Hz, ArH), 7.14 (d, 4H, J = 8.9 Hz, ArH), 4.12 (t, 4H, J = 6.4 Hz, CH_2), 2.51 (quin, 2H, J = 1.7, 1.9, 3.7 Hz, CH_2), 1.82 (m, 4H, CH_2); ^{13}C NMR (DMSO, 100MHz) δ 165.0, 163.3, 132.7, 130.5, 119.5, 115.0, 79.7, 79.3, 79.0, 68.3, 28.5; ν_{max} (Nujol) / cm^{-1} 3303 (N-H), 1660 (C=N-H), 1606 (Ar), 1509 (Ar), 1265 (C-O-C); *m/z* (ESP) 341 ([M+H]$^+$), found 341.1978, $C_{19}H_{25}N_4O_2$ requires 341.1975; anal. Found C 50.29 H 6.33 N 12.29, $C_{19}H_{30}Cl_2N_4O_4$ requires C 50.78, H 6.73, N 12.47.



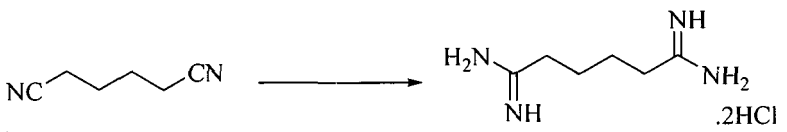
4-(5-(*p*-tolyloxy)pentyloxy)benzonitrile (14) Sodium (0.12 g, 5.22 mmol) was added portionwise to anhyd. EtOH (4.0 mL) and dissolved under a nitrogen atmosphere. To this a solution of 4-cyanophenol (0.57 g, 4.79 mmol) dissolved in dry ethanol (4.0 mL) was added followed by dropwise addition of 1,5 dibromopentane (0.65 mL, 4.79 mmol). The reaction mixture was stirred at reflux and monitored by TLC. After consumption of 4-cyanophenol the

reaction mixture was cooled to room temperature. In a separate flask, Na (0.57 g, 4.79 mmol) was added portionwise to dry EtOH (4.0 mL) stirring under nitrogen. To this a solution of *p*-cresol (0.5 mL, 4.79 mmol) in anhyd. EtOH (4.0 mL) was added and stirred for 10 minutes. This mixture was added dropwise to the cooled mixture and stirred under reflux for 3 days after which the mixture was cooled, filtered, the solid washed with water and dried under vacuum. Purification by column chromatography eluting with DCM: hexane (8:2) gave the desired compound **14** as a white solid (1.02 g, 72%). Mp 133°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.57 (d, 2H, J = 9.0 Hz, ArH), 7.07 (d, 2H, J = 8.6 Hz, ArH), 6.93 (d, 2H, J = 9.0 Hz, ArH), 6.79 (d, 2H, J = 8.6 Hz, ArH), 4.02 (t, 2H, J = 6.4 Hz, CH_2), 3.96 (t, 2H, J = 6.4 Hz, CH_2), 2.28 (s, 3H, CH_3), 1.86 (m, 4H, CH_2), 1.64 (m, 2H, CH_2); ^{13}C NMR ($CDCl_3$, 100MHz) δ 162.7, 157.2, 134.3, 130.3, 130.2, 115.5, 114.7, 104.1, 68.5, 68.0, 29.4, 29.1, 23.0, 20.8; ν_{max} (Nujol) / cm^{-1} 3322 (C-O-C), 3031 (Ar-H), 2921 (C-H), 2223 (C≡N), 1602 (Ar), 1506 (Ar), 1234 (C-O-C); *m/z* (CI) 313 ($[M+NH_4]^+$), found 313.19092, $C_{19}H_{25}N_2O_2$ requires 313.19162; anal. Found C 77.19, H 7.13, N 5.02, $C_{19}H_{21}NO_2$ requires C 77.26, H 7.17, N 4.74.

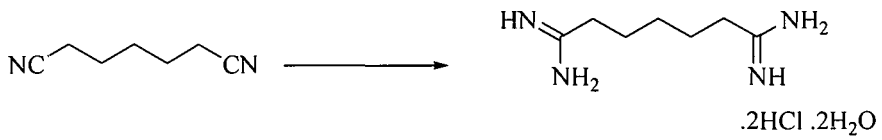


4-(5-(*p*-tolyloxy)pentyloxy)benzimidamide hydrochloride hydrate (15) Compound **14** (0.27 g, 0.91 mmol) was dissolved in a mixture of anhyd. benzene (100 mL) and anhyd. ethanol (1.60 mL), cooled to 0 ^{0}C and saturated with HCl gas. The mixture was sealed and stirred at room temperature for 3 days after which anhyd. Et_2O (20 mL) was introduced and the mixture stirred for 10 minutes. The solids were filtered under nitrogen and dissolved in a mixture of anhydrous EtOH (20 mL) and $EtOH.NH_3$ (20 mL). The mixture was heated overnight (50 ^{0}C), cooled to room temperature and reduced by half *in vacuo.* Ether (30 mL) was added to precipitate the solid which was filtered, washed and dried under vacuum. Purification by recrystallisation (2N HCl) gave the desired compound **15** as fine white needles (0.25 g, 75%). Mp 132°C; ^{1}H NMR (DMSO, 400 MHz) δ 7.84 (d, 2H, J = 9.1 Hz, ArH), 7.15 (d, 2H, J = 9.1 Hz, ArH), 7.06 (d, 2H, J

= 8.4 Hz, ArH), 6.80 (d, 2H, *J* = 8.4 Hz, ArH), 4.11 (t, 2H, *J* = 6.4 Hz, CH_2), 3.93 (t, 2H, *J* = 6.4 Hz, CH_2), 2.22 (s, 3H, CH_3), 1.78 (m, 4H, CH_2), 1.56 (m, 2H, CH_2); ^{13}C NMR (DMSO, 100MHz) δ 165.0, 163.3, 158.9, 130.5, 130.1, 115.1, 114.5, 79.5, 79.3, 79.0, 20.4; ν_{max} (Nujol) / cm^{-1} 3430 (NH), 3309 (C-O-C), 3093 (Ar-H), 1658 (C=N-H), 1606 (Ar), 1508 (Ar), 1245 (C-O-C); *m/z* (ESP) 313 ($[M+H]^+$), found 313.1916, $C_{19}H_{25}N_2O_2$ requires 313.1907; anal. Found C 62.19 H 7.42 N 7.66, $C_{19}H_{27}ClN_2O_3$ requires C 62.20, H 7.42, N 7.66.

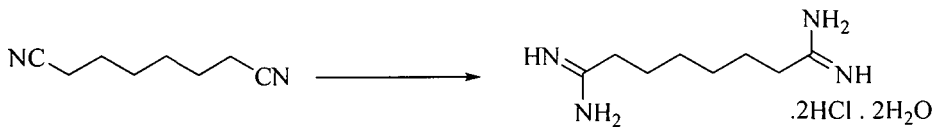


Adipimidamide dihydrochloride (16) 1,4 dicyanobutane (0.31 mL, 2.75 mmol) was suspended in a mixture of anhyd. benzene (25 mL) and ethanol (1.8 mL), cooled to 0 ^0C and saturated with HCl gas. The mixture was sealed and stirred at room temperature for 3 days after which anhyd. Et_2O (20 mL) was introduced and the mixture stirred for 10 minutes. The solids were filtered under nitrogen and dissolved in a mixture of anhyd. EtOH (21 mL) and anhyd. $EtOH.NH_3$ (21 mL). The mixture was heated overnight (50 ^0C), cooled to room temperature and reduced by half *in vacuo*. Ether (10 mL) was added to precipitate the solid which was filtered, washed and dried under vacuum. Purification by recrystallisation (2N HCl) gave the desired compound **16** as fine white needles (0.38 g, 64%). Mp 191°C; ^1H NMR (D_2O, 400 MHz) δ 2.52 (m, 4H, CH_2), 1.76 (m, 4H, CH_2); ^{13}C NMR (D_2O, 100MHz) δ 165.0, 32.0, 25.5; ν_{max} (Nujol) / cm^{-1} 3301 (N-H), 3079 (N-H), 2938 (C-H), 1677 (C=N); *m/z* (ESP) 143.1 ($[M+H]^+$); anal. Found C 33.54 H 7.50 N 25.80, $C_6H_{16}Cl_2N_4$ requires C 33.50, H 7.50, N 26.04.

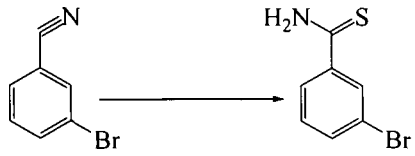


Heptane*bis*(imidamide) dihydrochloride dihydrate (17) 1,5 dicyanopentane (0.34 mL, 2.64 mmol) was suspended in a mixture of anhyd. benzene (24 mL) and EtOH (1.7 mL), cooled to 0 ^0C and saturated with HCl gas. The mixture was sealed and stirred at room temperature for 3 days after which anhyd Et_2O (20 mL) was introduced and the mixture stirred for 10 minutes. The

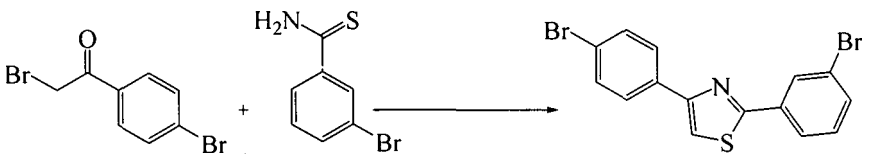
solids were filtered under nitrogen and dissolved in a mixture of anhyd. EtOH (21 mL) and anhyd. $EtOH.NH_3$ (21 mL). The mixture was heated overnight (50 ^0C), cooled to room temperature and reduced by half *in vacuo*. Ether (30 mL) was added to precipitate the solid which was filtered, washed and dried under vacuum. Purification by recrystallisation (2N HCl) gave the desired compound **17** as fine white needles (0.44 g, 63%); Mp 208°C; ^1H NMR (D_2O, 400 MHz) δ 2.50 (t, 4H, J = 7.8 Hz, CH_2), 1.73 (quin, 4H, J = 7.9, 15.4, 15.6 Hz, CH_2), 1.46 (quin, 2H, J = 6.7, 12.1, 15.2, CH_2); ^{13}C NMR ($CDCl_3$, 100MHz) δ 171.7, 32.2, 30.8, 27.3, 26.0; ν_{max} (Nujol) / cm^{-1} 3330 (N-H), 3075 (N-H), 2940 (C-H), 1678 (C=N); *m/z* (ESP) 157.1. ($[M+H]^+$); anal. Found C 32.50, H 7.77, N 22.51, $C_7H_{22}Cl_2N_4$ requires C 31.70, H 8.36, N 21.13.



Octane*bis*(imidamide) dihydrochloride (18) 1,5 dicyanohexane (0.30 mL, 2.14 mmol) was suspended in a mixture of anhyd. benzene (20 mL) and ethanol (1.4 mL), cooled to 0 ^0C and saturated with HCl gas. The mixture was sealed and stirred at room temperature for 3 days after which anhyd. Et_2O (20 mL) was introduced and the mixture stirred for 10 minutes. The solids were filtered under nitrogen and dissolved in a mixture of anhyd. EtOH (17 mL) and anhyd. $EtOH.NH_3$ (17 mL). The mixture was heated overnight (50 ^0C), cooled to room temperature and reduced by half *in vacuo*. Ether (30 mL) was added to precipitate the solid which was filtered, washed and dried under vacuum. Purification by recrystallisation (2N HCl) gave the desired compound **18** as fine white needles (0.33 g, 63%). Mp 201°C; ^1H NMR (D_2O, 400 MHz) δ 2.48 (t, 4H, J = 7.5 Hz, CH_2), 1.69 (m, 4H, CH_2), 1.40 (m, 4H, CH_2); ^{13}C NMR ($CDCl_3$, 100MHz) δ 165.1, 32.4, 27.8, 26.2; ν_{max} (Nujol) / cm^{-1} 3299 (N-H), 3074 (N-H), 2940 (C-H), 1677 (C=N); *m/z* (ESP) 171.1 ($[M+H]^+$); anal. Found C 38.43 H 8.28 N 22.77, $C_8H_{20}Cl_2N_4$ requires C 39.51, H 8.29, N 23.04.



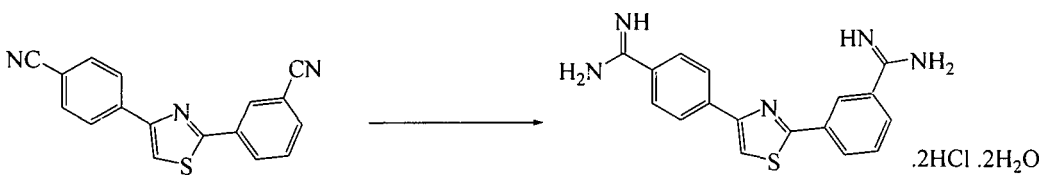
3-bromobenzothioamide (48a) Triethylamine (3.80 mL) was added to a solution of 3-bromobenzonitrile (5.02 g, 27.57 mmol) in pyridine (17 mL). The solution was cooled to 10°C and H_2S gas bubbled through for 15 min. The resulting green solution was stirred overnight. Nitrogen was bubbled through for 1 hr to remove any excess H_2S. Water (27 mL) was added and the mixture stirred for 10 min, a further portion of water (62 mL) was added and the pale yellow suspension left stirring overnight. The precipitate was filtered and rinsed with water to afford the title compound **48a** as bright yellow crystals (5.26 g, 88%). Mp 115-116°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.00 (t, 1H, *J* = 1.9 Hz, ArH), 7.80 (brs, 1H, NH), 7.77 (ddd, 1H, *J* = 1.8, 1.9, 7.9 Hz, ArH), 7.64 (ddd, 1H, *J* = 1.8, 1.9, 7.9 Hz, ArH), 7.28 (t, 1H, *J* = 7.8 Hz, ArH), 7.21 (brs, 1H, NH); ^{13}C NMR ($CDCl_3$, 100MHz) δ 201.4, 141.4, 135.1, 130.4, 130.4, 125.7, 123.0; ν_{max} (Neat) / cm^{-1} 3353 (N-H), 3158 (Ar-H), 1605 (Ar), 1502 (Ar), 1471 (C-N), 1247 (C=S), 892 (C=S); *m/z* (CI) 218 ($[M+H]^+$), found 215.94800, C_7H_7BrNS requires 215.94826; anal. Found C 38.63, H 2.74, N 6.42, C_7H_6BrNS requires C 38.91, H 2.80, N 6.48.



2-(3-bromophenyl)-4-(4-bromophenyl)thiazole (51) 2,4'-dibromoacetophenone (1.01 g, 3.63 mmol) was added to a solution of compound **48a** (790 mg, 3.66 mmol) in anhyd. EtOH (15 mL) and warmed to 45°C for 1 hr. The mixture was cooled to room temperature and stirred for 30min before filtering. The precipitate was washed with an EtOH : water mix (3:1, 10 ml) and dried to afford the title compound **51** as an off-white solid (1.30 g, 90%). Mp 166-167°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.20 (t, 1H, *J* = 1.9 Hz, ArH), 7.91 (ddd, 1H, *J* = 1.7, 1.7, 7.8 Hz, ArH), 7.86 (d, 2H, *J* = 8.8 Hz, ArH), 7.56 (m, 3H, ArH), 7.49 (s, 1H, CH), 7.32 (t, 1H, *J* = 7.7 Hz, ArH); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 166.2, 155.4, 135.4, 133.2, 133.0, 131.9, 130.4, 129.4,

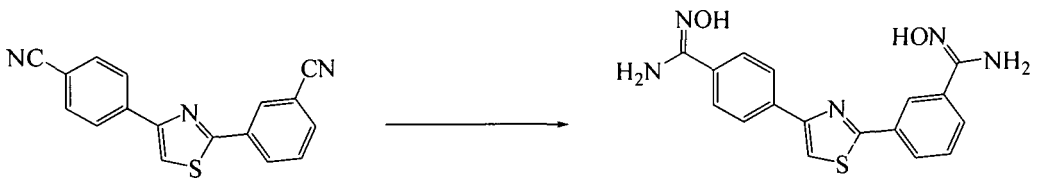
128.0, 125.2, 123.1, 122.4, 113.5; ν_{max} (Neat) / cm^{-1} 3070 (Ar-H), 2927 (C-H), 1587 (Ar), 1560 (C=N), 1508 (Ar), 744 (C-Br), 684 (C-S-C); *m/z* (ESP) 394/396/398 ($[M+H]^+$), found 393.8901, $C_{15}H_{10}{}^{79}Br_2NS$ requires 393.8917; anal. Found C 45.37, H 2.23, N 3.46, $C_{15}H_9Br_2NS$ requires C 45.59, H 2.29, N 3.54.

3,4'-(thiazole-2,4-diyl)dibenzonitrile (55) A suspension of compound **51** (1.02 g, 2.58 mmol) and CuCN (906 mg, 10.11 mmol) in anhyd. DMF (15 mL) were heated to reflux for 21 hrs. On cooling, the reaction mixture was poured into aqueous NH_4OH (10%, 50 ml) and extracted with $CHCl_3$ (100 mL). Both layers were filtered to remove the dark precipitate. The organic layer was washed with water (2 x 50 mL), brine (50 mL) and dried ($MgSO_4$). Removal of solvent gave a dark oily solid. Purification by column chromatography eluting with $CHCl_3$, afforded the title compound **55** as a pale solid (191 mg, 26%). Mp 199-200°C; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.36 (s, 1H, ArH), 8.24 (dt, 1H, J = 1.3, 8.0 Hz, ArH), 8.11 (d, 2H, J = 8.6 Hz, ArH), 7.74 (m, 3H, ArH), 7.70 (s, 1H, CH), 7.60 (t, 1H, J = 7.8 Hz, ArH); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 165.7, 154.7, 137.9, 134.3, 133.4, 132.7, 132.1, 130.5, 130.0, 129.9, 126.9, 126.6, 120.7, 118.7, 118.0, 116.2, 113.5, 111.8; ν_{max} (Neat) / cm^{-1} 3094 (Ar-H), 2996 (C-H), 2227 (C≡N), 1606 (Ar), 1509 (Ar), 676 (C-S-C); *m/z* (CI) 288 ($[M+H]^+$), found 288.0592, $C_{17}H_{10}N_3S$ requires 288.0595 anal. Found C 69.93, H 3.08, N 13.39, $C_{17}H_9N_3S$ requires C 71.06, H 3.16, N 14.62.



3,4'-(thiazole-2,4-diyl)dibenzimidamide (62) A suspension of compound **55** (0.35 g, 1.22 mmol) in anhyd. benzene (26 mL) and anhyd. EtOH (3.86 mL) was saturated with HCl gas and

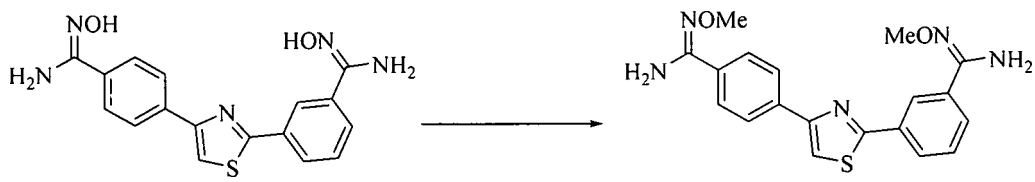
the solution left stirring at room temperature for 1 week. Anhydrous Et_2O was introduced and the precipitate was filtered under nitrogen and washed with anhyd. Et_2O. Anhydrous EtOH.NH_3 (26 mL) was added to a solution of the imidate in anhyd. EtOH (26 mL) and heated at reflux overnight. On cooling anhyd. Et_2O was added and the resulting precipitate filtered and washed with ether. Purification by recrystallisation (2N HCl) gave the desired compound **62** as fine white needles (0.26 g, 52%). Mp 370°C; ^{1}H NMR (DMSO, 400 MHz) δ 9.61 (s, 2H, NH_2), 9.48 (s, 2H, NH_2), 9.32 (s, 2H, NH_2), 9.20 (s, 2H, NH_2), 8.60 (s, 1H, CH), 8.47 (s, 1H, ArH), 8.41 (d, 1H, J = 7.8 Hz, ArH), 8.33 (d, 2H, J = 8.7 Hz, ArH), 7.98 (m, 3H, ArH), 7.80 (t, 1H, J = 7.8 Hz, ArH); ^{13}C NMR (DMSO, 100 MHz) δ 166.2, 165.5, 165.3, 154.0, 138.7, 133.6, 131.5, 130.4, 129.7, 129.2, 126.7, 126.2, 119.2; ν_{max} (Neat) / cm^{-1} 3035 (N-H), 1660 (C=N-H), 1595 (Ar), 1501 (Ar), 1232 (N=C-S), 671 (C-S-C); *m/z* (ESP) 322 ([M+H]$^+$), found 322.1126, $C_{17}H_{16}N_5S$ requires 322.1116; anal. Found C 49.49, H 4.64, N 17.12, $C_{17}H_{21}N_5SO_2Cl_2$ requires C 49.52, H 4.64, N 16.98.



***N'*-hydroxy-3-(4-(4-*N'*-hydroxycarbamimidoyl)phenyl)thiazole-2-yl)benzimidamide (63)**

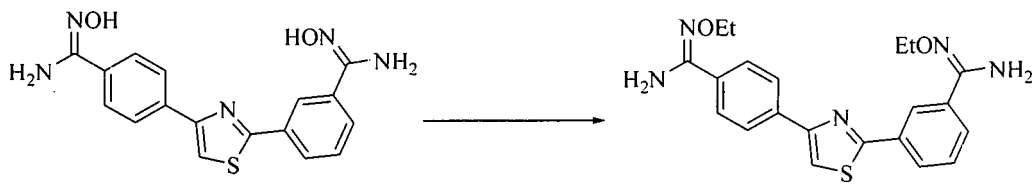
Potassium *tert* butoxide (0.78 g, 6.95 mmol) was added portionwise to a suspension of hydroxylamine hydrochloride (0.48 g, 6.90 mmol) in anhyd. DMSO (3.72 mL) at 5°C under an atmosphere of nitrogen. The mixture was stirred for 30min after which the mixture was added to compound **55** (0.20 g, 0.69 mmol) *via* cannula and the mixture stirred at room temperature overnight. The mixture was poured slowly onto ice water, filtered, washed with water, EtOH and dried yielding the title compound **63** as a white solid (0.24 g, 98%). Mp 203°C; ^{1}H NMR (DMSO, 400 MHz) δ 9.82 (s, 1H, NH), 9.72 (s, 1H, NH), 8.34 (s, 1H, ArH), 8.26 (s, 1H, CH), 8.06 (m, 3H, ArH), 7.81 (m, 3H, ArH), 7.55 (t, 1H, J = 7.8 Hz, ArH), 6.00 (s, 2H, NH_2), 5.88 (s, 2H, NH_2); ^{13}C NMR (DMSO, 100 MHz) δ 167.1, 155.1, 150.8, 150.6, 138.8, 134.7, 134.6, 133.3, 133.1, 129.5, 126.8, 123.4, 115.5; ν_{max} (Neat) / cm^{-1} 3432 (N-H), 3336 (N-O-H), 3066 (Ar-H), 1650 (C=N-O), 1602 (Ar), 1506 (Ar), 1240 (N=C-S), 671 (C-S-C); *m/z* (ESP) 354

($[M+H]^+$), found 354.1025, $C_{17}H_{16}N_5O_2S$ requires 354.1032; anal. Found C 57.80, H 4.27, N 19.94, $C_{17}H_{15}N_5O_2S$ requires C 57.78, H 4.28, N 19.82.



***N'*-methoxy-3-(4-(4-*N'*-methoxycarbamimidoyl)phenyl)thiazole-2yl)benzimidamide (65)**

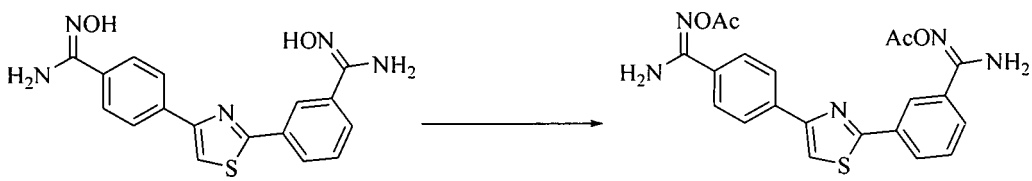
Dimethylsulphate (0.09 mL, 0.84 mmol) in dioxane (0.15 mL) was slowly added to a solution of compound **55** (0.10 g, 0.28 mmol) in dioxane (0.5 mL) and 2N NaOH (2.5 mL) at 0°C. The mixture was stirred for 2hrs from 0°C to room temperature after which the mixture was extracted with EtOAc (3x) and reduced *in vacuo*. The yellow residue was purified by column chromatography eluting with EtOAc : Petroleum ether (60:40) to afford the title compound **65** as a white solid (49 mg, 46%). Mp 175°C; ^{1}H NMR (DMSO, 400 MHz) δ 8.28 (s, 2H, CH, ArH), 8.08 (d, 3H, J = 8.5 Hz, ArH), 7.80 (m, 3H, ArH), 7.56 (t, 1H, J = 7.8 Hz, ArH), 6.26 (s, 2H, NH_2), 6.12 (s, 2H, NH_2), 3.79 (s, 3H, CH_3), 3.77 (s, 3H, CH_3); ^{13}C NMR (DMSO, 100 MHz) δ 167.0, 155.0, 151.0, 150.9, 135.0, 133.9, 133.2, 132.5, 129.6, 128.0, 127.4, 126.5, 126.2, 123.8, 115.9, 61.1, 61.0; ν_{max} (Neat) / cm^{-1} 3448 (N-O-C), 3305 (N-H), 1629 (C=N-O), 1240 (N=C-S), 682 (C-S-C); *m/z* (ESP) 382 ($[M+H]^+$), found 382.1338, $C_{19}H_{20}N_5O_2S$ requires 382.1319 anal. Found C 58.15, H 5.17, N 18.93, $C_{19}H_{19}N_5O_2S$ requires C 59.82, H 5.02, N 18.36.



***N'*-ethoxy-3-(4-(4-*N'*-ethoxycarbamimidoyl)phenyl)thiazole-2-yl)benzimidamide (66)**

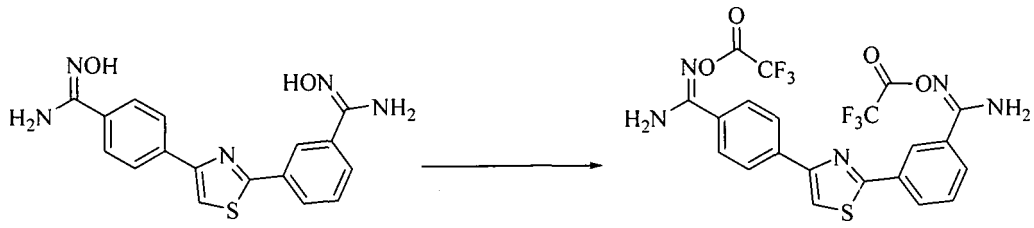
Diethylsulphate (0.11 mL, 0.84 mmol) in dioxane (0.14 mL) was slowly added to a solution of compound **55** (0.10 g, 0.28 mmol) in dioxane (0.42 mL) and 2N NaOH (2.26 mL) at 0°C. The mixture was stirred for 2hrs from 0°C to room temperature after which the mixture was extracted

with EtOAc (3 x 20mL) and reduced *in vacuo*. The yellow residue was purified by column chromatography eluting with EtOAc: Petroleum ether (60:40) yielding the title compound **66** as a white solid (52 mg, 45%). Mp 145°C; ^{1}H NMR (DMSO, 400 MHz δ 8.28 (s, 2H, ArH, CH), 8.08 (d, 3H, J = 8.5 Hz, ArH), 7.81 (m, 3H, ArH), 7.56 (t, 1H, J = 7.8 Hz, ArH), 6.20 (s, 2H, NH_2), 6.06 (s, 2H, NH_2), 4.01 (m, 4H, CH_2), 1.26 (m, 6H, CH_3); ^{13}C NMR (DMSO, 100 MHz) δ 167.0, 155.0, 150.9, 150.8, 134.9, 134.1, 133.2, 132.7, 129.5, 128.0, 127.4, 126.5, 126.2, 123.8, 115.9, 68.3, 68.2, 15.1; ν_{max} (Neat) / cm^{-1} 3459 (N-O-C), 3133 (N-H), 1623 (C=N-O), 1236 (N=C-S), 686 (C-S-C); *m/z* (ESP) 410 ([M+H]$^+$), found 410.1651, $C_{21}H_{24}N_5O_2S$ requires 410.1652; anal. Found C 62.00, H 5.80, N 17.00, $C_{21}H_{23}N_5O_2S$ requires C 61.59, H 5.66, N 17.10.

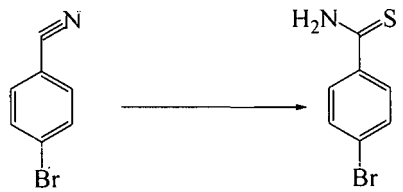


N'-acetoxy-3-(4-(4-N'-acetoxycarbamimidoyl)phenyl)thiazole-2-yl)benzimidamide (67)

Acetic anhydride (0.15 mL) was slowly added to a solution of compound **55** (0.15 g, 0.43 mmol) in glacial acetic acid (4.33 mL) at room temperature. The mixture was stirred overnight and poured slowly onto ice water, filtered, washed with water and dried yielding the title compound **67** as a white solid (0.17 g, 90%). Mp 193°C; ^{1}H NMR (DMSO, 400 MHz) δ 8.36 (s, 1H, CH), 8.34 (s, 1H, ArH), 8.16 (m, 3, ArH), 7.84 (m, 3H, ArH), 7.63 (t, 1H, J = 7.8 Hz, ArH), 7.02 (s, 2H, NH_2), 6.89 (s, 2H, NH_2), 2.16 (s, 6H, CH_3); ^{13}C NMR (DMSO, 100 MHz) δ 168.9, 168.8, 166.8, 156.4, 154.8, 136.0, 133.3, 133.0, 131.6, 129.8, 129.0, 128.9, 128.6, 124.7, 116.6, 20.24, 20.21; ν_{max} (Neat) / cm^{-1} 3612 (N-O-C), 3334 (N-H), 3035 (Ar-H), 2938 (C-H), 1739 (C=O), 1603 (Ar), 1226 (N=C-S), 678 (C-S-C); *m/z* (ESP) 460 ([M+Na]$^+$), found 460.1055, $C_{21}H_{19}N_5O_4{}^{23}NaS$ requires 460.1071; anal. Found C 57.70, H 4.39, N 15.99, $C_{21}H_{19}N_5O_4S$ requires C 57.66, H 4.38, N 16.01.

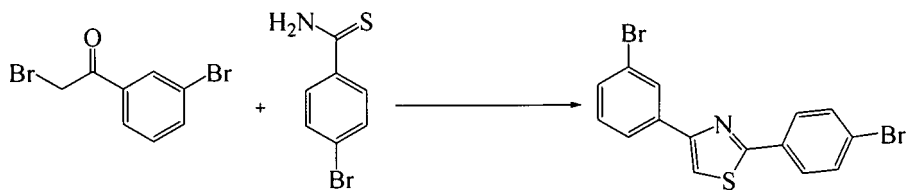


N'-(2,2,2-trifluoroacetoxy)-3-(4-(4-(N'-(2,2,2-trifluoroacetoxy)carbamimidoyl)phenyl)thiazol-2-yl)benzimidamide (68) Trifluoroacetic anhydride (0.14 mL, 1.0 mmol) was slowly added to a solution of compound **55** (0.10 g, 0.28 mmol) in glacial acetic acid (2.83 mL) at room temperature. The mixture was stirred overnight and poured slowly onto ice water, filtered, washed with water and dried yielding the title compound **68** as a white solid (0.10 g, 65%). Mp 198°C; ^{1}H NMR (DMSO, 400 MHz) δ 8.36 (s, 1H, CH), 8.34 (s, 1H, ArH), 8.17 (m, 3, ArH), 7.84 (m, 3H, ArH), 7.64 (t, 1H, J = 7.8 Hz, ArH), 7.02 (s, 2H, NH_2), 6.89 (s, 2H, NH_2), ^{13}C NMR (DMSO, 100 MHz) δ 174.7, 174.3, 168.9, 168.8, 166.8, 156.4, 154.8, 136.0, 133.3, 133.0, 131.6, 129.8, 129.0, 128.9, 128.6, 124.7, 116.6, 111.5, 111.2; *m/z* (ESP) 568 ([M+Na]$^+$), found 568.0490, $C_{21}H_{13}F_6N_5O_4{}^{23}NaS$ requires 568.0506; anal. Found C 46.04, H 2.51, N 12.92, $C_{21}H_{13}F_6N_5O_4S$ requires C 46.24, H 2.40, N 12.84.

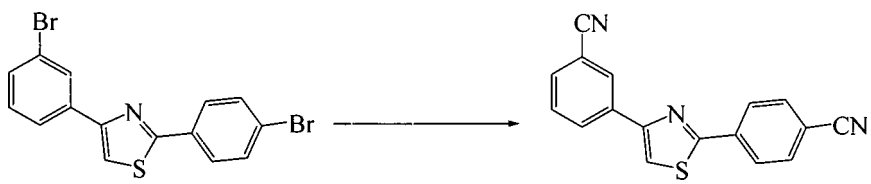


4-bromobenzothioamide (48b) Triethylamine (3.80 mL) was added to a solution of 4-bromobenzonitrile (5.01 g, 27.57 mmol) in pyridine (17 mL). The solution was cooled to 10°C and H_2S gas was bubbled through for 15 min. The resulting green solution was stirred overnight. Nitrogen was bubbled through for 1 hr to remove any excess H_2S. Water (27 mL) was added and the mixture stirred for 10 min, a further portion of water (62 mL) was added and the pale yellow suspension left stirring overnight. The precipitate was filtered and rinsed with water to afford the title compound **48b** as bright yellow crystals (5.52 g, 93%). Mp 115-116 °C; ^{1}H NMR (d_6-acetone, 400 MHz) δ 9.07 (bs, 1H, NH), 8.92 (s, 1H, NH), 7.94 (dd, 2H, J = 2.0, 6.5 Hz, ArH),

7.62 (dd, 2H, J = 2.0, 6.5 Hz, ArH); ^{13}C NMR (d_6-acetone, 100 MHz) δ 201.9, 140.2, 132.3, 130.4, 126.5; ν_{max} (Neat) / cm^{-1}3253 (N-H), 3149 (Ar-H), 1610 (Ar), 1509 (Ar), 1482 (C-N), 1257 (C=S), 883 (C=S); *m/z* (CI) 216 ([M]$^+$).

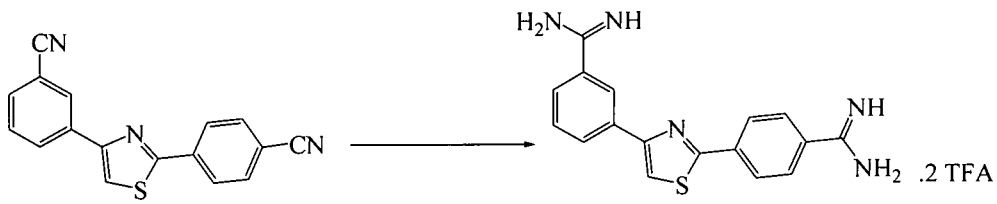


4-(3-bromophenyl)-2-(4-bromophenyl)thiazole (50) 2,3'-dibromoacetophenone (1.00 g, 3.59 mmol) was added to a solution of compound **48b** (777 mg, 3.60 mmol) in EtOH (15 mL) and warmed to 45°C for 1 hr. The mixture was cooled to room temperature and left for 30 min before filtering. The precipitate was washed with EtOH: water (3:1, 10 mL) and dried to afford thiazole **50** as an off white solid (1.03 g, 73%). Mp 168°C; ^{1}H NMR (d_6-acetone, 400 MHz) δ 8.30 (s, 1H, ArH), 8.15 (s, 1H, CH), 8.10 (s, 1H, ArH), 8.06 (d, 2H, J = 8.6 Hz, ArH), 7.74 (d, 2H, J = 8.6 Hz, ArH), 7.57 (dd, 1H, J = 1.0, 8.0 Hz, ArH), 7.45 (t, 1H, J = 7.6 Hz, ArH); ^{13}C NMR (d_6-acetone, 100 MHz) δ 166.1, 155.4, 135.2, 133.1, 131.9, 130.6, 129.5, 128.3, 125.0, 124.1, 123.1, 122.5, 113.5; ν_{max} (Neat) / cm^{-1} 3072 (Ar-H), 2926 (C-H), 1597 (Ar), 1560 (C=N), 1506 (Ar), 1232 (N=C-S), 744 (C-Br), 685 (C-S-C); *m/z* (CI) 396 ([M+H]$^+$).

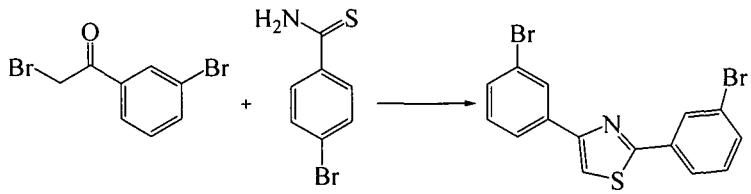


3,4'-(thiazole-2,4-diyl)dibenzonitrile (54) A suspension of compound **50** (978 mg, 2.50 mmol) and CuCN (895 mg, 10.0 mmol) in anhyd. DMF (15 mL) were heated to reflux for 21 hr. On cooling, the reaction mixture was poured into aqueous NH_4OH (10%, 50 mL) and extracted with $CHCl_3$ (100 mL). Both layers were filtered to remove the dark precipitate. The organic layer was washed with water (2 x 50 mL), brine (50 mL) and dried over $MgSO_4$. Removal of solvent gave a dark oily solid. Purification by column chromatography eluting with $CHCl_3$, afforded the title compound **54** as a pale solid (380 mg, 53%). Mp 200°C; ^{1}H NMR (d_6-acetone, 400 MHz) δ 8.36

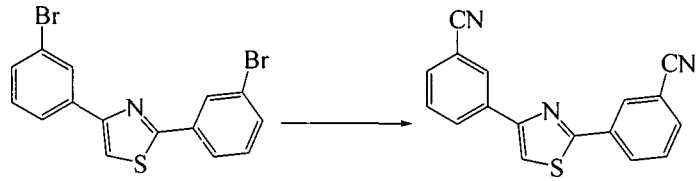
(d, 1H, J = 1.4 Hz, ArH), 8.30 (dt, 1H, J = 1.4, 8.0 Hz, ArH), 8.20 (s, 1H, CH), 8.19 (d, 2H, J = 8.6 Hz, ArH), 7.82 (d, 2H, J = 8.7 Hz, ArH), 7.66 (dt, 1H, J = 1.3, 7.6 Hz, ArH), 7.59 (t, 1H, J = 7.8 Hz, ArH); ^{13}C NMR (d_6-acetone, 100 MHz) δ 165.4, 154.7, 134.3, 132.9, 131.9, 131.3, 131.1, 128.4, 119.6, 119.3, 118.6, 114.3; ν_{max} (Neat) / cm^{-1} 3089 (Ar-H), 2948 (C-H), 2225 (C≡N), 1602 (Ar), 1503 (Ar), 678 (C-S-C); *m/z* (CI) 288 ([M+H]$^+$).



3,4'-(thiazole-2,4-diyl)dibenzimidamide (58) A suspension of compound **54** (310 mg, 1.08 mmol) in anhyd. benzene (23mL) and anhyd. EtOH (3.4 mL) was saturated with HCl gas and the solution left stirring at room temperature for 1 week. Anhydrous ether was added to the precipitate which was filtered under N_2 and washed with anhyd. Et_2O. Anhydrous EtOH. NH_3 was added to a solution of the imidate in EtOH (23 mL) and heated to reflux overnight. On cooling, anhyd. Et_2O was added and the resulting precipitate filtered and washed with Et_2O. Crude yield (249 mg, 72%). The crude product (100 mg) was purified by reverse phase HPLC using a YMC-pack ODS-A column (250 x 20 mm I.D, 5 μM) eluting with CH_3CN: Water 0.1% TFA (20-80 % gradient over 20 min). Removal of solvent afforded the TFA salt of the desired compound **58** as a colourless solid (33 mg, 17%). Mp 257-258°C; ^{1}H NMR (DMSO, 400 MHz) δ 9.48 (bs, 6H, NH, NH_2), 8.52 (s, 1H, CH), 8.49 (s, 1H, ArH), 8.43 (d, 1H, J = 7.8 Hz, ArH), 8.30 (d, 2H, J = 8.4 Hz, ArH), 8.01 (d, 2H, J = 8.4 Hz, ArH), 7.85 (d, 1H, J = 7.8 Hz, ArH), 7.76 (t, 1H, J = 7.8 Hz, ArH); ^{13}C NMR (DMSO, 100 MHz) δ 166.1, 166.0, 165.4, 159.2, 158.9, 154.5, 137.4, 134.7, 131.4, 130.0, 129.9, 129.6, 129.4, 128.2, 126.9, 126.1, 118.4; ν_{max} (Neat) / cm^{-1} 3050 (N-H), 1652 (C=N-H), 1602 (Ar), 1496 (Ar), 682 (C-S-C); *m/z* (ESP) 322 ([M+H]$^+$), found 322.1132, $C_{17}H_{16}N_5S$ requires 322.1126; anal. Found C 46.17, H 3.04, N 12.81, $C_{21}H_{17}N_5O_4F_6S$ requires C 45.91, H 3.11, N 12.74.

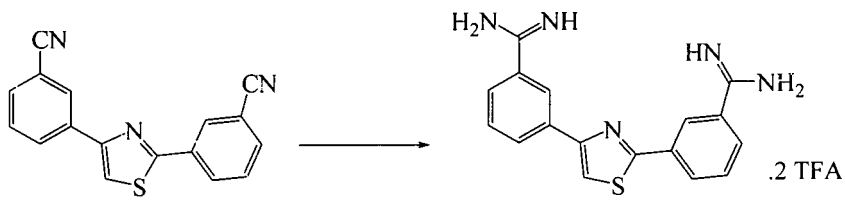


2,4-*bis*(3-bromophenyl)thiazole (52) 2,3'-dibromoacetophenone (1.01 g, 3.63 mmol) was added to a solution of compound **48b** (777 mg, 3.60 mmol) in EtOH (15 mL) and warmed to 45°C for 1 hr. The mixture was cooled to room temperature and left for 30 min before filtering. The precipitate was washed with EtOH: water (3:1, 10 mL) and dried to afford thiazole **52** as a colourless solid (1.23 g, 86%). Mp 174°C; ^{1}H NMR (d_6-acetone, 400 MHz) δ 8.30 (m, 2H, ArH), 8.15 (s, 1H, CH), 8.10 (d, 1H, J = 7.8 Hz, ArH), 8.06 (d, 1H, J = 7.8 Hz, ArH), 7.70 (dd, 1H, J = 1.0, 8.0 Hz, ArH), 7.57 (dd, 1H, J = 1.0, 8.0 Hz, ArH), 7.51 (t, 1H, J = 8.0 Hz, ArH), 7.45 (t, 1H, J = 7.8 Hz, ArH); ^{13}C NMR (d_6-acetone, 100 MHz) δ 167.2, 137.8, 134.4, 134.3, 132.4, 132.3, 132.0, 130.4, 130.2, 130.1, 126.7, 126.4, 124.0, 123.8, 113.6; ν_{max} (Neat) / cm^{-1} 3074 (Ar-H), 2926 (C-H), 1597 (Ar), 1560(C=N), 1506 (Ar), 744 (C-Br), 686 (C-S-C); *m/z* (CI) 396 ([M+H]$^+$).

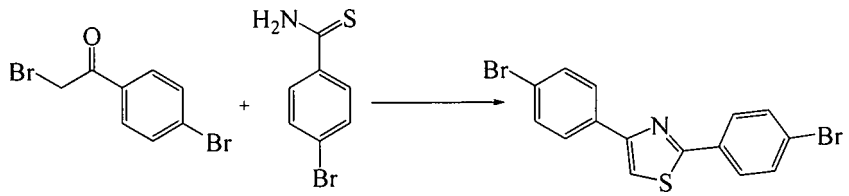


3,3'-(thiazole-2,4-diyl)dibenzonitrile (56) A suspension of compound **52** (1.00 g, 2.53 mmol) and CuCN (906 mg, 10.12 mmol) in anhyd. DMF (15 mL) were heated to reflux for 21 hr. On cooling, the reaction mixture was poured into aqueous NH_4OH (10%, 50 mL) and extracted with $CHCl_3$ (100 mL). Both layers were filtered to remove the dark precipitate. The organic layer was washed with water (2 x 50 mL), brine (50 mL) and dried ($MgSO_4$). Removal of solvent gave a dark oily solid. Purification by column chromatography eluting with EtOAc: hexane (2:8) afforded the title compound **56** as a pale solid (394 mg, 54%). Mp 204-205°C; ^{1}H NMR (d_6-acetone, 400 MHz) δ 8.58 (s, 1H, ArH), 8.49 (m, 2H, ArH), 8.37 (s, 1H, CH), 7.97 (dt, 1H, J = 1.10, 6.3 Hz ArH), 7.81 (m, 4H, ArH); ν_{max} (Neat) / cm^{-1} 3097 (Ar-H), 2904 (C-H), 2231 (C≡N), 1609 (Ar), 1505 (Ar), 676 (C-S-C); *m/z* (CI) 288 ([M+H]$^+$).

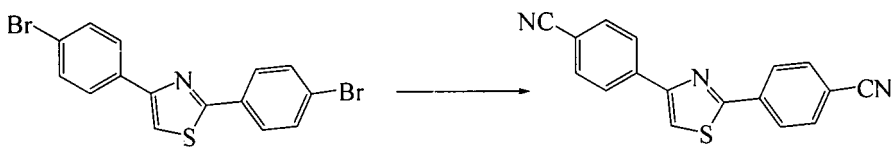
Preparation of 0.67M Alkylchloroaluminium amide Solutions. Trimethyl aluminium (10 mL, 2M in toluene) was added dropwise to a suspension of ammonium hydrochloride (1.07 g, 0.02M) in anhyd. toluene (20 mL) under N_2. The reaction was warmed to room temperature and stirred until gas (CH_4) evolution had ceased. The solution can be stored at 4°C for up to 48 hr.



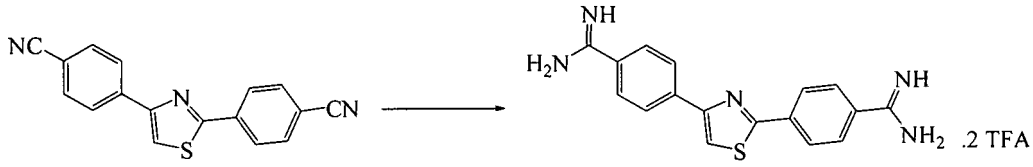
3,3'-(thiazole-2,4-diyl)dibenzimidamide (60) The freshly prepared alkyl chloroaluminium reagent (5.2 mL, 0.67M, 3.48 mmol) was added to compound **56** (100 mg, 0.35 mmol) in anhyd. toluene (1 mL) and heated to 80°C overnight under a nitrogen atmosphere. On cooling, the aluminium complex was decomposed by pouring into a slurry of silica gel (2.0 g) in $CHCl_3$. The mixture was stirred for 5 min, filtered and the solid washed with MeOH (20 mL), after which the filtrate was reduced *in vacuo* giving the crude amidine as a pale solid in quantitative yield. The crude product (100 mg) was purified by reverse phase HPLC using a YMC-pack ODS-A column (250 x 20 mm I.D, 5 μM) eluting with CH_3CN: Water 0.1% TFA (20-80 % gradient over 20 min). Removal of solvent afforded the desired compound **60** as a colourless solid (38 mg, 22%). Mp 246°C; ^{1}H NMR (DMSO, 400 MHz) δ 9.50 (m, 6H, NH, NH_2), 8.51 (s, 1H, CH), 8.43 (m, 4H, ArH), 7.96 (d, 1H, J = 8.0 Hz, ArH), 7.80 (m, 3H, ArH); ^{13}C NMR (DMSO, 100 MHz) δ 166.2, 166.1, 165.8, 158.9, 154.2, 134.8, 133.7, 131.4, 130.4, 130.2, 130.0, 129.9, 129.5, 128.2, 126.3, 126.1, 119.0, 117.8, 116.0; *m/z* (ESP) 322 ($[M+H]^+$), found 322.1141, $C_{17}H_{16}N_5S$ requires 322.1126; anal. Found C 46.13, H 3.25, N 12.13, $C_{21}H_{17}N_5O_4F_6S$ requires C 45.91, H 3.12, N 12.74.



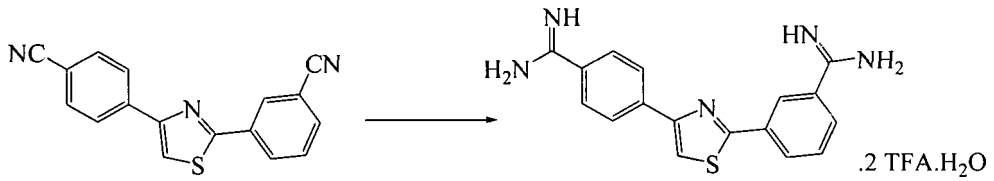
2,4-*bis*(4-bromophenyl)thiazole (53) 2,4'-dibromoacetophenone (1.00 g, 3.60 mmol) was added to a solution of compound **48b** (777 mg, 3.60 mmol) in EtOH (15 mL) and warmed to 45°C for 1 hr. The mixture was cooled to room temperature and left for 30 min before filtering. The precipitate was washed with EtOH: water (3:1, 10 mL) and dried to afford thiazole **53** as a pale solid (1.33 g, 94%). Mp 175-176°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.89 (d, 2H, *J* = 8.0 Hz, ArH), 7.85 (d, 2H, *J* = 8.0 Hz, ArH), 7.59 (d, 2H, *J* = 5.5 Hz, ArH), 7.56 (d, 2H, *J* = 5.5 Hz, ArH), 7.47 (s, 1H, CH); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 166.5, 156.0, 135.2, 133.1, 132.0, 129.6, 128.5, 125.2, 124.3, 123.2, 112.7; ν_{max} (Neat) / cm^{-1} 3072 (Ar-H), 2926 (C-H), 1590 (Ar), 1560 (C=N), 1509 (Ar), 747 (C-Br), 684 (C-S-C); *m/z* (CI) 396 ([M+H]$^+$).



4,4'-(thiazole-2,4-diyl)dibenzonitrile (57) A suspension of compound **53** (1.00 g, 2.53 mmol) and CuCN (906 mg, 10.12 mmol) in anhyd. DMF (15 ml) were heated to reflux for 21 hr. On cooling, the reaction mixture was poured into aqueous NH_4OH (10%, 50 mL) and extracted with $CHCl_3$ (100 mL). Both layers were filtered to remove the dark precipitate. The organic layer was washed with water (2 x 50 mL), brine (50 mL) and dried ($MgSO_4$). Removal of solvent gave a dark oily solid. Purification by column chromatography eluting with $CHCl_3$, afforded the title compound **57** as a pale solid (361 mg, 50%). Mp 200°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.14 (d, 2H, *J* = 8.3 Hz, ArH), 8.10 (m, 2H, ArH), 7.75 (m, 4H, ArH), 7.26 (s, 1H, CH); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 166.5, 137.4, 133.2, 133.1, 130.1, 127.4, 127.3, 127.2, 119.1, 118.6, 117.1, 114.2, 112.4; ν_{max} (Neat) / cm^{-1} 3093 (Ar-H), 2987 (C-H), 2225 (C≡N), 1602 (Ar), 1501 (Ar), 669 (C-S-C); *m/z* (CI) 288 ([M+H]$^+$).

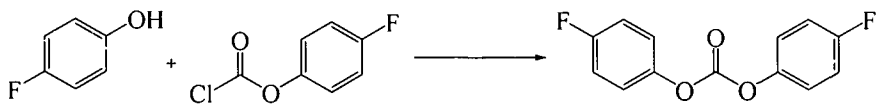


4,4'-(thiazole-2,4-diyl)dibenzimidamide (61) The freshly prepared alkylchloroaluminium reagents (4 mL, 0.67 M, 2.7 mmol) was added to compound **57** (78 mg, 0.27 mmol) in anhyd. toluene (1 mL) and heated to 80 °C overnight under nitrogen. On cooling, the aluminium complex was decomposed by pouring into a slurry of silica gel (2.00 g) in $CHCl_3$. The mixture was stirred for 5 min before filtering and washing the solid with MeOH (20 mL). Removal of solvent *in vacuo* gave the crude amidine as a pale solid in quantitative yield. The crude product (100 mg) was purified by reverse phase HPLC using a YMC-pack ODS-A column (250 x 20 mm I.D, 5 μM) eluting with CH_3CN: Water 0.1% TFA (20-80% gradient over 20 min). Removal of solvent afforded the desired compound as an off-white solid (38 mg, 22%). Mp 271-272°C; ^1H NMR (DMSO, 400 MHz) δ 9.32 (bs, 6H, NH , NH_2), 8.54 (s, 1H, CH), 8.25 (d, 2H, J = 8.5 Hz, ArH), 8.22 (d, 2H, J = 8.5 Hz, ArH), 7.92 (d, 2H, J = 8.5 Hz, ArH), 7.90 (d, 2H, J = 8.5 Hz, ArH); ^{13}C NMR (DMSO, 100 MHz) δ 165.5, 165.4, 154.4, 138.7, 137.4, 129.6, 129.2, 127.9, 126.9, 126.8, 119.6; *m/z* (ESP) 322 ($[M+H]^+$), found 322.1121, $C_{17}H_{16}N_5S$ requires 322.1126; anal. Found C 46.05, H 3.10, N 12.44, S 5.77, $C_{21}H_{17}N_5O_4F_6S$ requires C 45.91, H 3.12, N 12.74, S 5.83.

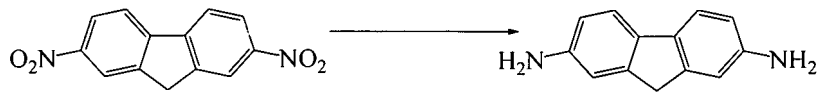


3,4'-(thiazole-2,4-diyl)dibenzimidamide (59) A suspension of compound **55** (300 mg, 1.04 mmol) in anhyd. benzene (22 mL) and anhyd. EtOH (3.3 mL) was saturated with HCl gas and the solution left stirring at room temperature for 1 week. Anhydrous Et_2O was added to the precipitate which was filtered under N_2 and washed with anhyd. Et_2O. Anhydrous EtOH. NH_3 was added to a solution of the imidate in EtOH (22 mL) and heated to reflux overnight (17hr). On cooling, anhyd. Et_2O was added and the resulting precipitate filtered and washed with Et_2O.

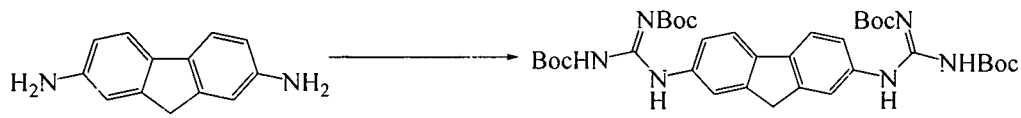
Crude yield (249 mg, 72%). The crude product (100 mg) was purified by reverse phase HPLC using a YMC-pack ODS-A column (250 x 20 mm I.D, 5 μM) eluting with CH_3CN: Water 0.1% TFA (20-80 % gradient over 20 min). Removal of solvent *in vacuo* afforded the TFA salt of compound **59** as a colourless solid (61 mg, 31%). Mp 258°C; ^{1}H NMR (DMSO, 400 MHz) δ 9.48 (m, 6H, NH , NH_2), 8.58 (s, 1H, CH), 8.46 (t, 1H, J = 1.5 Hz, ArH), 8.42 (dt, 1H, J = 1.0, 8.0 Hz, ArH), 8.33 (d, 2H, J = 8.5 Hz, ArH), 7.98 (d, 2H, J = 8.5 Hz, ArH), 7.94 (s, 1H, ArH), 7.81 (t, 1H, J = 8.0 Hz, ArH); ^{13}C NMR (DMSO, 100 MHz) δ 165.7, 165.5, 164.2, 159.1, 158.8, 157.6, 154.0, 138.8, 133.7, 131.5, 130.5, 130.3, 129.9, 129.2, 127.8, 126.8, 126.3, 119.1; *m/z* (ESP) 322 ($[M+H]^+$), found 322.1150, $C_{17}H_{16}N_5S$ requires 322.1126; anal. Found C 44.54, H 3.44, N 12.12, $C_{21}H_{19}N_5O_5F_6S$ requires C 45.45, H 3.37, N 12.34.



***Bis*(4-fluorophenyl)carbonate (76)** 4-fluorophenyl chloroformate (1.02 g, 5.84 mmol) in DCM (2.2 mL) was added to a solution of 4-fluorophenol (0.65 g, 5.84 mmol) in pyridine (0.47 mL). The mixture was stirred at 0°C for 15 min after which the mixture was stirred at room temperature overnight. DCM (20 mL) was added to the reaction mixture and washed with water followed by 0.5N NaOH, brine, water, dried (Na_2SO_4) and reduced *in vacuo*. Purification by column chromatography eluting with $CHCl_3$, afforded the compound **76** as a white solid (1.20 g, 82%), Mp 126°C; ^{1}H NMR (DMSO, 400 MHz) δ 7.23 (m, 4H, ArH), 7.09 (m, 4H, ArH); ^{13}C NMR (DMSO, 100 MHz) δ 162.1, 159.6, 152.6, 147.2, 147.1, 122.8, 122.7, 116.8, 116.5; *m/z* (CI) 268 ($[M+NH_4]^+$), found 268.07910, $C_{13}H_{12}F_2O_3N$ requires 268.07852; anal. Found C 62.43, H 3.21, $C_{13}H_8F_2O_3$ requires C 62.41, H 3.22.

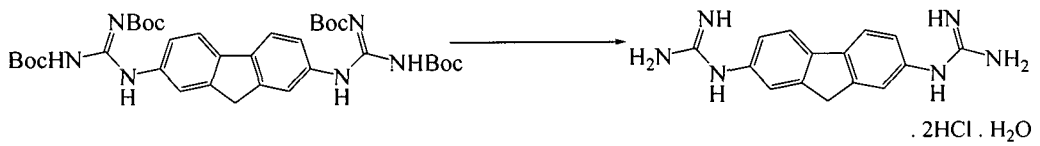


9*H*-fluorene-2,7-diamine (106) To a suspension of 2,7-dinitro-*9H*-fluorene (0.12 g, 0.46 mmol) and zinc (0.98 g, 15.08 mmol) in EtOH (4.0 mL) was added calcium chloride (33 mg, 0.29 mmol) in water (50 μL). The mixture was stirred for 2 hrs at reflux, filtered hot under reduced pressure, washed with excess hot EtOH and the filtrate reduced. Purification by column chromatography eluting with (DCM: MeOH 8:2) afforded the compound **106** as a red solid (69 mg, 76%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.40 (d, 2H, J = 8.2 Hz, ArH), 6.82 (s, 2H, ArH), 6.67 (d, 1H, J = 2.1 Hz, ArH), 6.65 (d, 1H, J = 2.3 Hz, ArH), 3.71 (s, 2H, CH_2), 3.64 (brs, 2H, NH_2), 1.54 (brs, 2H, NH_2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 144.7, 144.4, 134.0, 119.6, 114.2, 112.4, 37.1; ν_{max} (Neat) / cm^{-1} 3561 (N-H), 3039 (Ar-H), 2942 (C-H), 1614 (Ar), 1495 (Ar); *m/z* (CI) 197 ([M+H]$^+$), found 197.10836, $C_{13}H_{12}N_2$ requires 197.10788; anal. Found C 79.20, H 6.19, N 13.99, $C_{13}H_{12}N_2$ requires C 79.56, H 6.16, N 14.27.

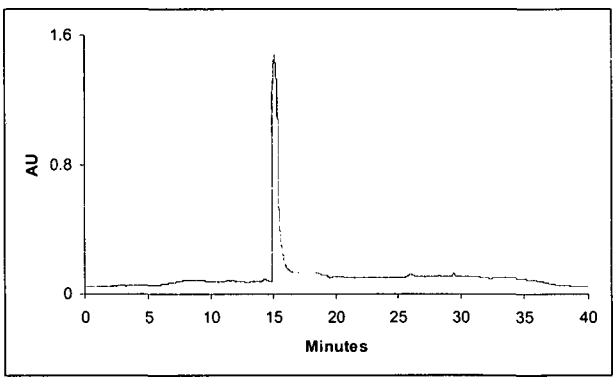


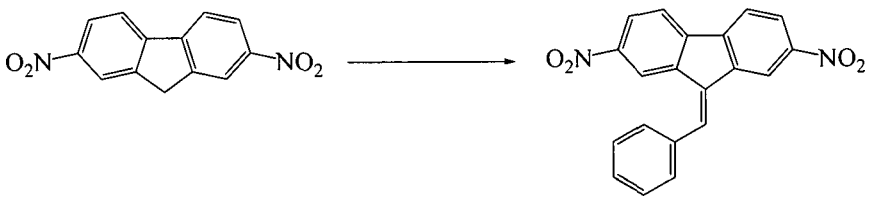
***tert*-butyl-(9*H*-fluorene-2,7-diyl)*bis*(azanediyl)*bis*((*tert*-butoxycarbonylamino)methan-1-yl-1-ylidene)dicarbamate (107)** Compound **106** (0.1 g, 0.51 mmol), triethylamine (0.43 mL, 3.06 mmol), 1,3-*bis*(*tert*-butoxycarbonyl)-2-methyl-thiopseudourea (0.31 g, 1.07 mmol), and mercury (II) chloride (0.32 g, 1.17 mmol) were suspended in anhyd. DMF (3 mL) and stirred overnight under a nitrogen atmosphere. After which the mixture was diluted with DCM (100 mL), washed with Na_2SO_3 and filtered through a pad of celite. The filtrate was washed with water, brine, dried over Na_2SO_4, filtered and reduced *in vacuo*. The resultant solid was recrystallised from DCM/MeOH after which purification by column chromatography eluting with DCM afforded compound **107** as a white solid (0.21 g, 60%); Mp >330°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 11.67 (s, 2H, NH), 10.42 (s, 2H, NH), 7.89 (s, 2H, ArH), 7.64 (d, 2H, J = 8.2 Hz, ArH), 7.49 (d, 1H, J = 1.8 Hz, ArH), 7.47(d, 1H, J = 1.8 Hz, ArH), 3.90 (s, 2H, CH_2), 1.52 (m, 36H, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 164.0, 153.8, 144.6, 138.5, 135.7, 121.3, 120.1, 119.3, 84.0, 80.0, 37.6,

31.2, 28.6, 28.5; *m/z* (ESP) 681 ($[M+H]^+$), found 681.3612, $C_{35}H_{49}N_6O_8$ requires 681.3636; anal. Found C 61.10, H 6.97, N 12.19, $C_{35}H_{48}N_6O_8$ requires C 61.75, H 7.11, N 12.34.

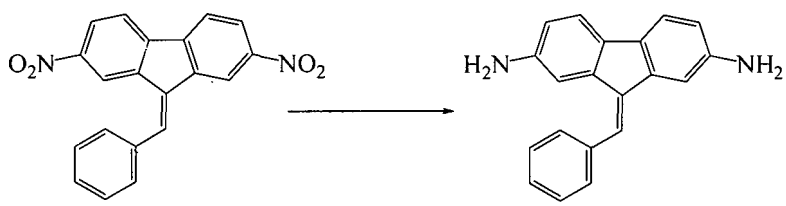


1, 1'-(*9H*-fluorene-2,7-diyl)diguanidine (108) Compound **107** (0.13 g, 0.19 mmol) was suspended in a mixture of anhyd. DCM (1.83 mL) and anhyd. EtOH (1.22 mL), cooled to 0°C and saturated with gaseous hydrogen chloride. The mixture was sealed and stirred at room temperature for 4 days after which the mixture was reduced *in vacuo* and the residue crystallised with EtOH/Et_2O yielding compound **108** as a grey solid (41 mg, 41%); Mp >320°C; ^1H NMR ($CDCl_3$, 400 MHz) δ 10.16 (brs, 2H, NH), 7.97 (d, 2H, J = 8.1 Hz, ArH), 7.54 (brs, 8H, NH), 7.47 (s, 2H, ArH), 7.26 (d, 2H, J = 8.1 Hz, ArH), 3.97 (s, 2H, CH_2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 156.5, 144.9, 139.0, 134.2, 123.8, 121.8, 121.3, 36.9; *m/z* (ESP) 281 ($[M+H]^+$), found 281.1515, $C_{15}H_{17}N_6$ requires 281.1519; anal. Found C 48.28, H 4.93, N 21.21, $C_{15}H_{20}Cl_2N_6O$ requires C 48.53, H 5.43, N 22.64. Reverse-Phase HPLC;



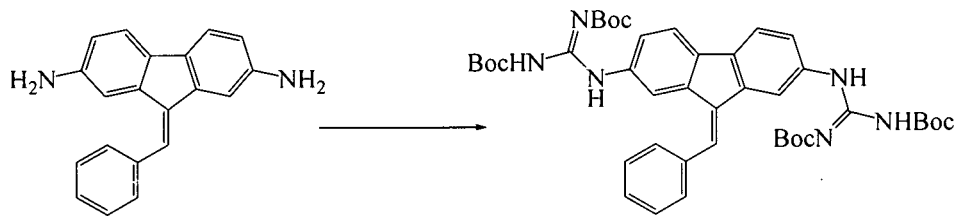


9-benzylidene-2,7-dinitro-9*H*-fluorene (81) To a suspension of 2,7-dinitro-*9H*-fluorene (1.00 g, 3.90 mmol) and KF:Al_2O_3 (0.65 g, 4.68 mmol) in anhydrous DMF (16 mL) was added benzaldehyde (0.44 mL, 4.29 mmol), after which the mixture was stirred overnight at room temperature. The resulting suspension was filtered under reduced pressure, washed with an excess of DCM and reduced *in vacuo* furnishing **81** as a flocculent yellow solid (0.95 g, 71%). Mp >240°C (decomp); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.75 (d, 1H, *J* = 2.1 Hz, ArH), 8.57 (d, 1H, *J* = 2.1 Hz, ArH), 8.37 (dd, 1H, *J* = 1.9 , 8.4 Hz, ArH), 8.30 (dd, 1H, *J* = 2.1, 8.5 Hz, ArH), 8.07 (s, 1H, CH), 7.98 (d, 1H, *J* = 8.3 Hz, ArH), 7.96 (d, 1H, *J* = 8.5 Hz, ArH), 7.63 (m, 2H, ArH), 7.56 (m, 3H, ArH); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 154.2, 148.4, 148.0, 143.9, 141.9, 141.5, 138.3, 134.6, 133.7, 133.4, 129.9, 129.2, 129.1, 124.2, 123.8, 121.3, 121.2, 119.9, 116.2; ν_{max} (Neat) / cm^{-1} 3003 (Ar-H), 2927 (C-H), 1601 (Ar), 1503 (Ar), 1324 (N=O), 1230 (C-N); anal. Found C 69.55, H 3.44, N 8.19, $C_{20}H_{12}N_2O_4$ requires C 69.76, H 3.51, N 8.13.

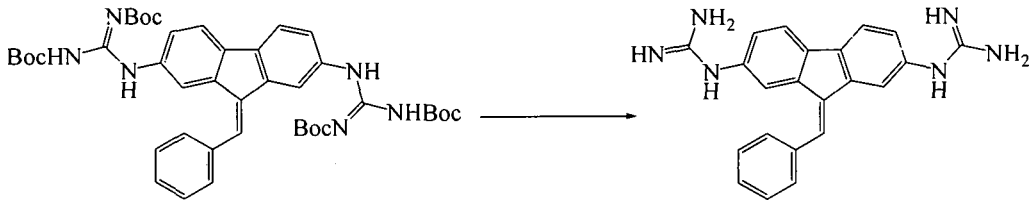


9-benzylidene-9*H*-fluorene-2,7-diamine (87) To a suspension of compound **81** (0.15 g, 0.44 mmol) and zinc (0.94 g, 14.41 mmol) in EtOH (4.0 mL) was added calcium chloride (31 mg, 0.28 mmol) in water (50 μL). The mixture was stirred for 2 hrs at reflux and filtered hot under reduced pressure, washed with excess hot EtOH and the filtrate reduced. Purification by column chromatography eluting with DCM:MeOH (8:2) afforded compound **81** as a red solid (87 mg, 69%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.54 (d, 2H, *J* = 7.8 Hz, ArH), 7.49 (s, 1H, CH), 7.42 (t,

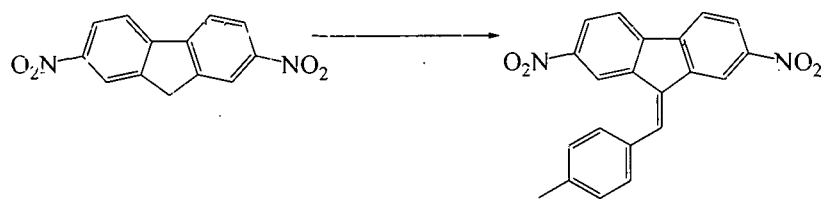
2H, J = 7.8 Hz, ArH), 7.33 (m, 3H, ArH), 7.02 (d, 1H, J =2.1 Hz, ArH), 6.80 (d, 1H, J = 2.1 Hz, ArH), 6.65 (dd, 1H, J = 2.1, 8.0 Hz, ArH), 6.58 (dd, 1H, J = 2.1, 8.0 Hz, ArH), 3.54 (brs, 3H, NH), 1.65 (brs, 1H, NH); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 145.0, 144.4, 141.0, 138.0, 137.5, 137.3, 134.0, 132.0, 129.9, 129.5, 128.8, 128.2, 126.6, 119.5, 116.0, 115.9, 115.1, 114.5, 112.4, 112.0, 107.6; *m/z* (ESP) 285 ([M+H]$^+$), found 285.1392, $C_{20}H_{17}N_2$ requires 285.1406; anal. Found C 83.29, H 5.75, N 9.38, $C_{20}H_{16}N_2$ requires C 84.47, H 5.67, N 9.85.



^{tert}-butyl(9-benzylidene-9*H*-fluorene-2,7-diyl)*bis*(azanediyl)*bis*((^{tert}-butoxycarbonylamino)-methan-1-yl-1ylidene) dicarbamate (93) Compound **87** (0.15 g, 0.52 mmol) triethylamine (0.43 mL, 3.12 mmol), 1,3-*bis*(*tert*-butoxycarbonyl)-2-methyl-thiopseudourea (0.31 g, 1.09 mmol), and mercury (II)chloride (0.32 g, 1.19 mmol) were suspended in anhyd. DMF (3.2 mL) and stirred overnight under an atmosphere of nitrogen. The mixture was diluted with DCM (120 mL), washed with Na_2SO_3 and filtered through a pad of celite. The filtrate was washed with water (3x), brine (2x), dried over Na_2SO_4 filtered and reduced *in vacuo*. The solid was recrystallised from DCM/MeOH after which purification by column chromatography eluting with DCM afforded compound **93** as an orange solid (0.26 g, 65%). Mp >290°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 11.63 (s, 2H, NH), 10.32 (m, 2H, NH), 8.12 (s, 1H, CH), 7.76 (s, 2H, ArH), 7.57 (m, 7H, ArH), 7.44 (d, 1H, J = 8.2 Hz, ArH), 7.28 (d, 1H, J = 7.9 Hz, ArH), 1.51 (m, 36H, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 153.9, 153.8, 147.1, 140.6, 140.5, 137.8, 135.6, 130.5, 130.2, 126.4, 125.9, 125.3, 124.2, 123.1, 122.1, 120.4, 120.2, 120.1, 119.7, 114.8, 84.1, 48.8, 39.3, 28.6; *m/z* (ESP) 769 ([M+H]$^+$), found 769.3925, $C_{42}H_{53}N_6O_8$ requires 769.3939; anal. Found C 64.80, H 6.74, N 10.44, $C_{42}H_{52}N_6O_8$ requires C 65.61, H 6.82, N 10.93.

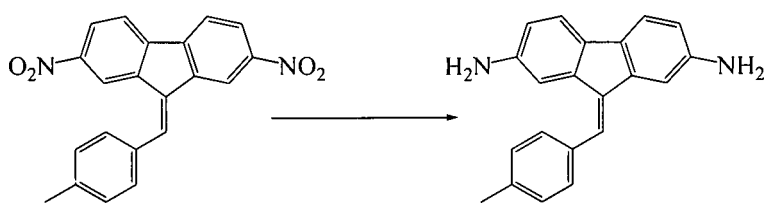


1,1'-(9-benzylidene-9*H*-fluorene-2,7-diyl)diguanidine (99) Compound **93** (0.21 g, 0.27 mmol) was suspended in a mixture of dry DCM (3 mL) and EtOH (2 mL), cooled to 0°C and saturated with gaseous hydrogen chloride. The mixture was sealed and stirred at room temperature for 4 days after which the mixture was reduced *in vacuo* and the residue crystallised with $EtOH/Et_2O$ yielding the title compound **99** as a grey solid (95 mg, 72%). Mp >320°C; ^1H NMR ($CDCl_3$, 400 MHz) δ 10.15 (s, 1H, NH), 10.04 (s, 1H, NH), 9.96 (s, 1H, NH), 8.05 (s, 1H, CH), 7.97 (d, 1H, J = 8.2 Hz, ArH), 7.95 (d, 1H, J = 8.2 Hz, ArH), 7.92 (d, 1H, J = 8.2 Hz, ArH), 7.88 (s, 1H, ArH), 7.69 (d, 1H, J = 7.4 Hz, ArH), 7.50 (m, 5H, NH), 7.25 (m, 5H, ArH), 7.13 (s, 1H, ArH); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.4, 156.7, 156.5, 156.4, 148.5, 136.4, 134.5, 134.2, 134.1, 129.7, 129.6, 129.1, 128.5, 124.3, 121.7, 121.3, 120.4, 118.1, 79.7; *m/z* (ESP) 369 ($[M+H]^+$), found 369.1828, $C_{22}H_{21}N_6$ requires 369.1813.

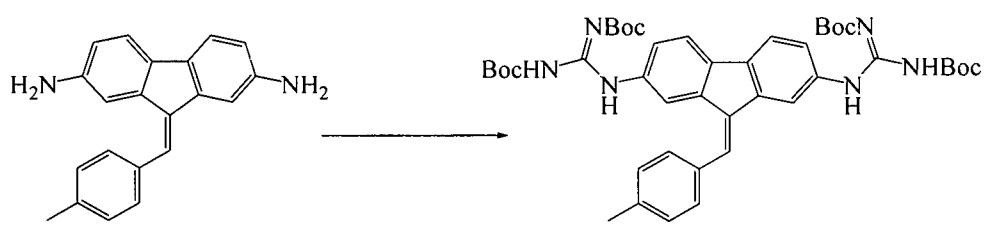


9-(4-methylbenzylidene)-2,7-dinitro-9*H*-fluorene (82) To a suspension of 2,7-dinitro-*9H*-fluorene (1.5 g, 5.85 mmol) and $KF:Al_2O_3$ (0.98 g, 7.00 mmol) in anhyd. DMF (16 mL) was added *p*-tolualdehyde (0.69 mL, 6.43 mmol) and the suspension was stirred overnight at room temperature. The resulting mixture was filtered under reduced pressure, washed with an excess of DCM and reduced *in vacuo* furnishing **82** as a flocculent yellow solid. (1.61 g, 77%). Mp >240°C (decomp); ^1H NMR ($CDCl_3$, 400 MHz) δ 8.73 (d, 1H, J = 1.9 Hz, ArH), 8.70 (d, 1H, J = 2.1Hz, ArH), 8.35 (dd, 1H, J = 1.9, 8.4 Hz, ArH), 8.30 (dd, 1H, J = 2.1, 8.4 Hz, ArH), 8.03 (s, 1H, CH), 7.96 (dd, 2H, J = 2.8, 8.3 Hz, ArH), 7.55 (d, 2H, J = 8.0 Hz, ArH), 7.37 (d, 2H, J = 8.0

Hz, ArH), 2.49 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 145.5, 134.5, 130.2, 129.8, 129.7, 129.5, 123.8, 121.2, 116.1, 21.8; anal. Found C 70.70, H 3.99, N 6.97, $C_{21}H_{14}N_2O_4$ requires C 70.38, H 3.93, N 7.82.

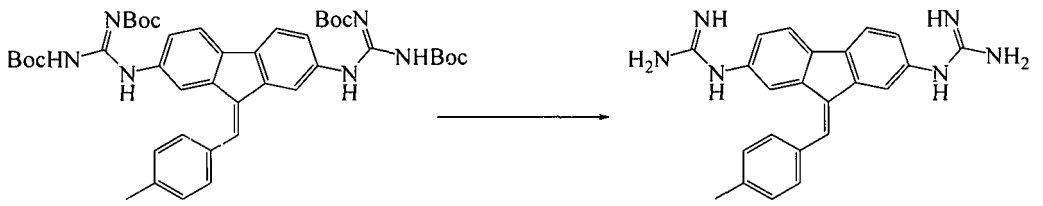


9-(4-methylbenzylidene)-9*H*-fluorene-2,7-diamine (88) To a suspension of compound **82** (0.20 g, 0.55 mmol) and zinc (1.18 g, 18.01 mmol) in EtOH (5 mL) was added calcium chloride (39.28 mg, 0.35 mmol) in water (60 μL). The mixture was stirred for 2 hrs at reflux and filtered hot under reduced pressure, washed with excess hot EtOH and the filtrate reduced. Purification by column chromatography eluting with DCM:MeOH (8:2) afforded compound **88** as a red solid. (0.12 g, 73%). ^1H NMR ($CDCl_3$, 400 MHz) δ 7.46 (m, 3H, ArH, CH), 7.32 (dd, 2H, J = 3.0, 8.0 Hz, ArH), 7.23 (m, 2H, ArH), 7.03 (d, 1H, J = 1.9 Hz, ArH), 6.93 (d, 1H, J = 2.1 Hz, ArH), 6.65 (dd, 1H, J = 2.1, 8.0 Hz, ArH), 6.59 (dd, 1H, J = 2.1, 8.0 Hz, ArH), 3.59 (brs, 4H, NH), 2.42 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 148.3, 144.8, 138.1, 134.0, 129.6, 126.8, 119.6, 119.5, 115.9, 115.7, 114.5, 114.2, 112.4, 111.9, 107.5, 21.7, 21.5; *m/z* (ESP) 299 ($[M+H]^+$), found 299.1548, $C_{21}H_{19}N_2$ requires 299.1563; anal. Found C 84.65, H 6.26, N 8.72, $C_{21}H_{18}N_2$ requires C 84.53, H 6.08, N 9.39.

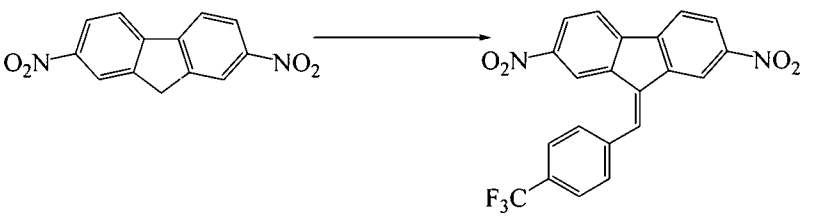


^{tert}-butyl(9-(4-methylbenzylidene)-9*H*-fluorene-2,7-diyl)*bis*(azanediyl)*bis*((^{tert}butoxycarbonylamino)methan-1-yl-1-ylidene)dicarbamate (94) Compound **88** 61mg (0.20 mmol), triethylamine (0.17 mL, 1.22 mmol), 1,3-*bis*(*tert*-butoxycarbonyl)-2-methyl-thiopseudourea

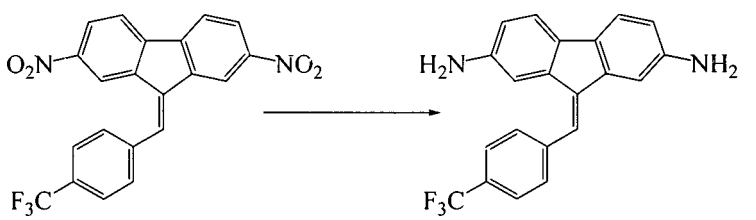
(0.12 g, 0.42 mmol), and mercury (II) chloride (0.13 g, 0.46 mmol) were suspended in anhyd. DMF (1.22 mL) and stirred overnight under a nitrogen atmosphere. The mixture was diluted with DCM (100 mL), washed with Na_2SO_3 and filtered through a pad of celite. The filtrate was washed with water, brine, dried over Na_2SO_4 filtered and reduced *in vacuo*. After which the solid was recrystallised from DCM/MeOH, subsequent purification by column chromatography eluting with DCM afforded title compound **94** as an orange solid (97 mg, 62%). Mp >300°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 11.67 (brs, 2H, NH), 10.41 (s, 2H, NH), 7.89 (s, 1H, CH), 7.64 (d, 2H, J = 8.3 Hz, ArH), 7.56 (m, 5H, ArH), 7.48 (d, 2H, J = 8.4 Hz, ArH), 7.30 (d, 1H, J = 8.0 Hz, ArH), 1.53 (m, 36H, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 154.7, 153.9, 147.6, 146.0, 141.2, 140.6, 137.6, 135.2, 130.2, 129.9, 128.3, 126.6, 125.9, 124.8, 123.8, 122.2, 121.4, 121.1, 119.4, 82.3, 39.6 28.6, 28.5; *m/z* (ESP) 805 ($[M+Na]^+$), found 805.3887, $C_{43}H_{54}N_6O_8{}^{23}Na$ requires 805.3901; anal. Found C 65.97, H 6.95, N 10.73, $C_{43}H_{54}N_6O_8$ requires C 65.10, H 7.16, N 10.30.



1,1'-(9-(4-methylbenzylidene)-9*H*-fluorene-2,7-diyl)diguanidine (100) Compound **94** (97 mg, 0.12 mmol) was suspended in a mixture of dry DCM (1.15 mL) and EtOH (0.76 mL), cooled to 0°C and saturated with gaseous hydrogen chloride. The mixture was sealed and stirred at room temperature for 4 days after which the mixture was reduced *in vacuo* and the residue crystallised with EtOH/Et_2O yielding compound **100** as an orange solid (10 mg, 16%). Mp >320°C; ^{1}H NMR (DMSO, 400 MHz) δ 7.97 (d, 2H J = 8.2 Hz, ArH), 7.51 (m, 14H, ArH, NH, CH), 7.26 (dd, 2H, J = 1.9, 8.2 Hz, ArH), 7.12 (m, 1H, ArH), 2.50 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 156.5, 148.6, 144.9, 139.1, 134.2, 129.6, 129.1, 123.8, 121.9, 121.3, 36.9, 34.7, 21.0; *m/z* (ESP) 385 ($[M+H]^+$), found 385.2141 $C_{23}H_{25}N_6$ requires 385.2130.

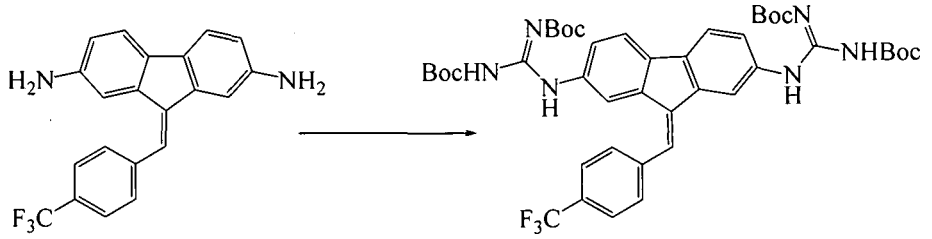


9-(4-trifluoromethylbenzylidene)-2,7-dinitro-9*H*-fluorene (83) To a suspension of 2,7-dinitro-*9H*-fluorene (1.00 g, 3.90 mmol) and $KF:Al_2O_3$ (0.65 g, 4.68 mmol) in anhyd. DMF (16 mL) was added *p*-trifluorotolualdehyde (0.52 mL, 4.29 mmol), and the mixture was stirred overnight at room temperature. The resulting suspension was filtered under reduced pressure, washed with an excess of DCM and reduced *in vacuo* furnishing **83** as a flocculent yellow solid. (1.16 g, 72%). Mp >245°C (decomp); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.74 (d, 1H, J = 1.9 Hz, ArH), 8.40 (d, 1H, J = 1.7 Hz, ArH), 8.38 (d, 1H, J = 1.9 Hz, ArH), 8.32 (dd, 1H, J = 1.9, 8.5 Hz, ArH), 8.01 (s, 1H, CH), 7.98 (dd, 2H, J = 3.6, 8.4 Hz, ArH), 7.84 (d, 2H, J = 8.4 Hz, ArH), 7.75 (d, 2H, J = 8.0 Hz, ArH); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 145.1, 134.5, 130.1, 129.9, 125.1, 123.8, 121.8, 120.2, 118.0, 116.7; ν_{max} (Neat) / cm^{-1} 3097 (Ar-H), 2944 (C-H), 1608 (Ar), 1505 (Ar), 1339 (N=O), 1162 (C-F), 1120 (C-F); anal. Found C 61.03, H 2.68, N 6.74, $C_{21}H_{11}N_2O_4F_3$ requires C 61.17, H 2.69, N 6.79.

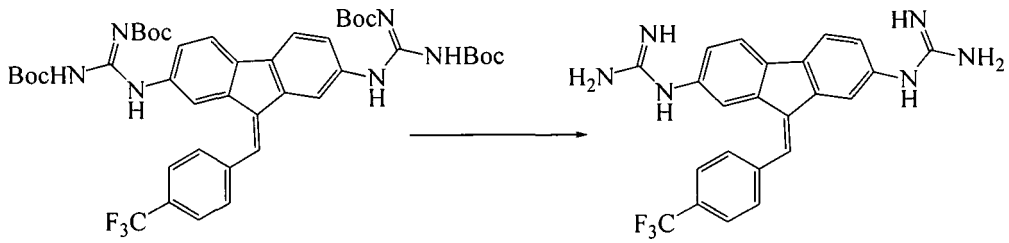


9-(4-trifluoromethylbenzylidene)-9*H*-fluorene-2,7-diamine (89) To a suspension of compound **83** (0.12 g, 0.29 mmol) and zinc (0.62 g, 9.50 mmol) in EtOH (4 mL) was added calcium chloride (21 mg, 0.19 mmol) in water (30 μL). The mixture was stirred for 2 hrs at reflux and filtered hot under reduced pressure, washed with excess hot EtOH and the filtrate reduced. Purification by column chromatography eluting with DCM:MeOH (8:2) afforded title compound **89** as a red solid (82 mg, 80%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.53 (d, 2H, J = 8.0 Hz, ArH), 7.43 (s, 1H, CH), 7.36 (d, 1H, J = 8.0 Hz, ArH), 7.32 (dd, 2H, J = 2.3, 8.0 Hz, ArH),

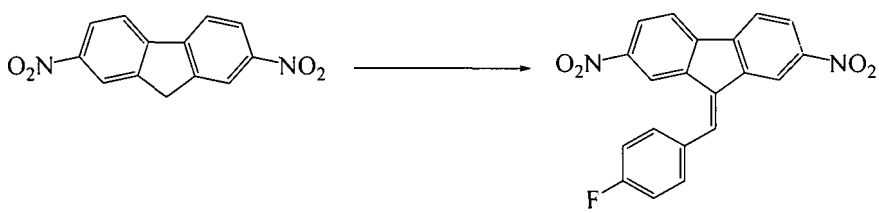
7.02 (d, 1H, J = 2.1 Hz, ArH), 6.64 (m, 3H, ArH), 6.45 (d, 1H, J = 1.9 Hz, ArH), 3.62 (brs, 4H, NH_2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 147.4, 146.4, 144.7, 141.5, 134.9, 134.3, 130.2, 129.7, 124.4, 124.3, 119.8, 119.7, 114.7, 114.2; *m/z* (ESP) 353 ($[M+H]^+$), found 353.1258, $C_{21}H_{16}N_2F_3$ requires 353.1266; anal. Found C 71.62, H 4.65, N 7.43, $C_{21}H_{15}N_2F_3$ requires C 71.58, H 4.29, N 7.95.



^{tert}-butyl(9-(4-trifluoromethylbenzylidene)-9*H*-fluorene-2,7 diyl)*bis*(azanediyl) *bis* ((^{tert}-butoxycarbonylamino)methan-1-yl-1-ylidene)dicarbamate (95) Compound **89** (0.18 g, 0.53 mmol), triethylamine (0.44 mL, 3.16 mmol), 1,3-*bis*(*tert*-butoxycarbonyl)-2-methyl-thiopseudourea (0.32 g, 1.11 mmol), and mercury (II) chloride (0.33 g, 1.22 mmol) were suspended in anhyd. DMF (3.30 mL) and stirred overnight under a nitrogen atmosphere. The mixture was diluted with DCM (110 mL), washed with Na_2SO_3 and filtered through a pad of celite. The filtrate was washed with water, brine, dried over Na_2SO_4, filtered and reduced *in vacuo*. The solid was recrystallised from DCM/MeOH after which purification by column chromatography eluting with DCM afforded the title compound **95** as a yellow solid (0.29 g, 65%). Mp >339°C; ^1H NMR ($CDCl_3$, 400 MHz) δ 11.65 (s, 2H, NH), 10.41 (s, 2H, NH), 8.09 (s, 1H, CH), 7.77 (d, 2H, J = 1.8 Hz, ArH), 7.58 (m, 6H, ArH), 7.49 (d, 2H, J = 8.2 Hz, ArH), 1.52 (m, 36H, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 154.3, 153.9, 147.3, 142.1, 140.6, 137.7, 135.5, 130.2, 126.4, 125.6, 124.3, 123.4, 122.2, 122.1, 119.3, 82.2, 39.5, 28.6. *m/z* (ESP) 859 ($[M+Na]^+$), found 859.3611, $C_{43}H_{51}F_3N_6O_8{}^{23}Na$ requires 859.3627.

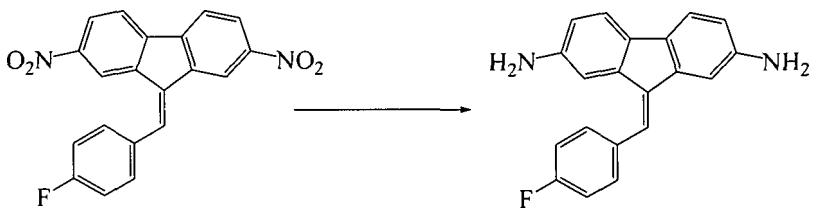


1,1'-(9-(4-trifluoromethylbenzylidene)-9*H*-fluorene-2,7-diyl)diguanidine (101) Compound **95** (0.15 g, 0.18 mmol) was suspended in a mixture of dry DCM (1.66 mL) and EtOH (1.10 mL), cooled to 0°C and saturated with gaseous hydrogen chloride. The mixture was sealed and stirred at room temperature for 4 days after which the mixture was reduced *in vacuo* and the residue crystallised with EtOH/Et_2O yielding title compound **101** as a pale yellow solid (57 mg, 58%). Mp >320°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 10.17 (brs, 2H, NH), 8.10 (s, 1H, CH), 7.90 (m, 3H, ArH), 7.61 (d, 1H, J = 8.2 Hz, ArH), 7.54 (brs, 8H, NH, ArH), 7.45 (d, 1H, J = 8.2 Hz, ArH), 7.35 (s, 1H, ArH), 7.27 (m, 2H, ArH); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 156.7, 156.5, 148.1, 140.6, 138.3, 135.9, 134.8, 134.5, 134.2, 130.6, 130.4, 129.1, 126.0, 125.2, 124.4, 121.7, 121.6, 121.4; *m/z* (ESP) 437 ($[M+H]^+$), found 437.1702, $C_{23}H_{20}N_6F_3$ requires 437.1702.

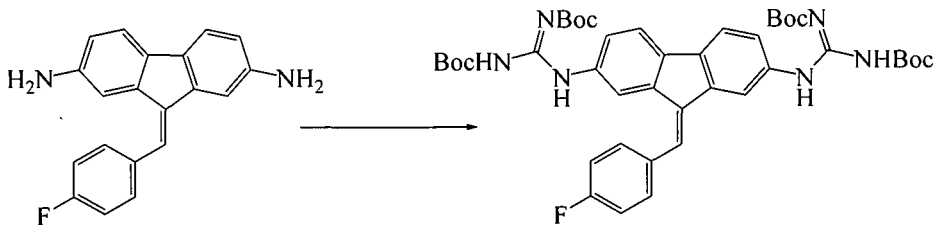


9-(4-fluorobenzylidene)-2,7-dinitro-9*H*-fluorene (84) To a suspension of 2,7-dinitro-*9H*-fluorene (1.01 g, 3.94 mmol) and KF:Al_2O_3 (0.65 g, 4.68 mmol) in anhyd. DMF (16 mL) was added *p*-fluorobenzaldehyde (0.45 mL, 4.29 mmol) and the mixture was stirred overnight at room temperature. The resulting suspension was filtered under reduced pressure, washed with an excess of DCM and reduced *in vacuo* furnishing **84** as a flocculent yellow solid (1.12 g, 79%). Mp >319°C (decomp); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.73 (s, 1H, ArH), 8.56 (s, 1H, ArH), 8.37 (d, 1H, J = 8.2 Hz, ArH), 8.31 (d, 1H, J = 8.2 Hz, ArH), 8.01 (s, 1H, CH), 7.97 (m, 4H, ArH), 7.63 (m, 2H, ArH); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 164.1, 138.5, 133.4, 132.9, 131.8, 131.2,

123.4, 121.1, 119.2, 118.9, 116.5; ν_{max} (Neat) / cm^{-1} 3106 (Ar-H), 2908 (C-H), 1600 (Ar), 1509 (Ar), 1332 (N=O), anal; Found C 65.96, H 2.98, N, 7.70, $C_{20}H_{11}FN_2O_4$ requires C 66.30, H 3.06, N 7.73.

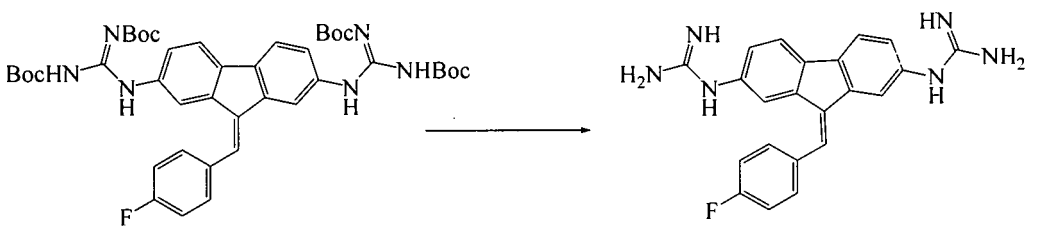


9-(4-fluorobenzylidene)-9*H*-fluorene-2,7-diamine (90) To a suspension of compound **84** (0.12 g, 0.33 mmol) and zinc (0.71 g, 10.85 mmol) in EtOH (4 mL) was added calcium chloride (24 mg, 0.21 mmol) in water (4 μL). The mixture was stirred for 2 hrs at reflux and filtered hot under reduced pressure, washed with excess hot EtOH and the filtrate reduced. Purification by column chromatography eluting with DCM:MeOH (8:2) afforded title compound **90** as a red solid (82 mg, 82%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.60 (d, 2H, J = 7.8 Hz, ArH), 7.55 (d, 2H, J = 7.8 Hz, ArH), 7.45 (s, 1H, CH), 7.34 (m, 2H, ArH), 6.63 (m, 4H, ArH), 3.59 (brs, 4H, NH_2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 160.2, 147.5, 147.1, 144.6, 138.4, 134.8, 134.3, 131.1, 124.4, 123.5, 121.1, 119.2, 118.9, 114.5; ν_{max} (Neat) / cm^{-1} 3482 (N-H), 3049 (Ar-H), 2919 (C-H), 1596 (Ar), 1504 (Ar); anal. C 78.82, H 4.62, N, 8.98, $C_{20}H_{15}N_2F$ requires C 79.45, H 5.00, N 9.27.



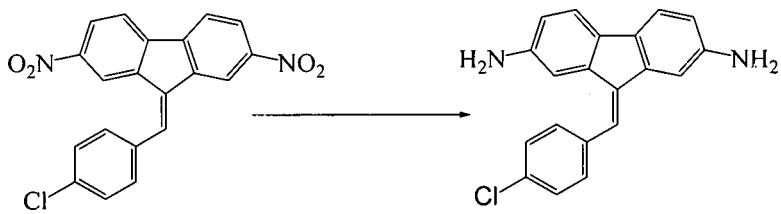
^{tert}-butyl(9-(4-fluorobenzylidene)-9*H*-fluorene-2,7-diyl)*bis*(azanediyl)*bis*((^{tert}-butoxy carbonylamino)methan-1-yl-1-ylidene)dicarbamate (96) Compound **90** (0.15 g, 0.49 mmol), triethylamine (0.41 mL, 2.94 mmol), 1,3-*bis*(*tert*-butoxycarbonyl)-2-methyl-thiopseudourea (0.30 g, 1.03 mmol), and mercury (II) chloride (0.31 g, 1.13 mmol) were suspended in anhyd.

DMF (3.15 mL) and stirred overnight under a nitrogen atmosphere. The mixture was diluted with DCM (120 mL), washed with Na_2SO_3 and filtered through a pad of celite. The filtrate was washed with water, brine, dried over Na_2SO_4, filtered and reduced *in vacuo*. The solid was recrystallised from DCM/MeOH after which purification by column chromatography eluting with DCM afforded title compound **96** as a yellow solid (0.25 g, 65%). Mp >300°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 11.65 (s, 2H, NH), 10.45 (s, 1H, NH), 10.14 (s, 1H, NH), 8.07 (s, 1H, CH), 7.79 (d, 1H, J = 1.7, ArH), 7.60 (m, 6H, ArH), 7.36 (dd, 1H, J = 1.9, 8.2 Hz, ArH), 7.18 (m, 2H, ArH), 1.52 (m, 36H, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 161.7, 154.3, 153.8, 146.3, 140.9, 138.6, 137.3, 136.3, 136.2, 135.8, 132.6, 131.8, 131.7, 127.4, 123.9, 122.7, 120.3, 120.1, 120.0, 116.0, 115.8, 114.6, 80.0, 28.6, 28.5; anal. Found C 63.90, H 6.60, N 10.60, $C_{42}H_{51}N_6O_8F$ requires C 64.11, H 6.53, N 10.68.

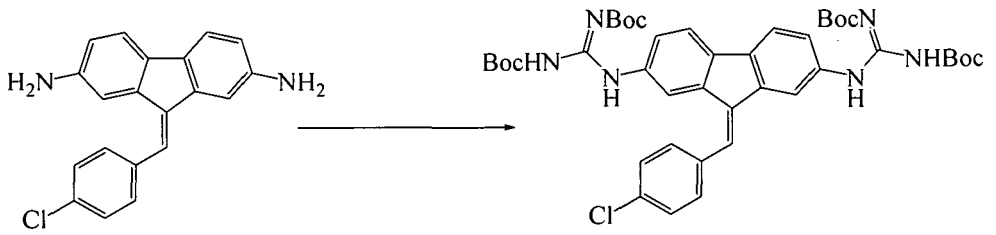


1,1'-(9-(4-fluorobenzylidene)-9*H*-fluorene-2,7-diyl)diguanidine (102) Compound **96** (0.19 g, 0.25 mmol) was suspended in a mixture of dry DCM (2.0 mL) and EtOH (1.5 mL), cooled to 0 °C and saturated with gaseous hydrogen chloride. The mixture was sealed and stirred at room temperature for 4 days after which the mixture was reduced *in vacuo* and the residue crystallised with EtOH/Et_2O yielding title compound **102** as a yellow solid (89 mg, 72%). Mp >320°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.01 (s, 1H, CH), 7.96 (d, 1H, J = 8.0 Hz, ArH), 7.95 (d, 1H, J = 8.0 Hz, ArH), 7.86 (d, 1H, J = 1.7 Hz, ArH), 7.76 (d, 1H, J = 8.4 Hz, ArH), 7.74 (d, 1H, J = 8.3 Hz, ArH), 7.55 (m, 8H, NH), 7.43 (d, 1H, J = 1.9 Hz, ArH), 7.37 (d, 1H, J = 8.7 Hz, ArH), 7.35 (d, 1H, J = 8.9 Hz, ArH), 7.27 (m, 2H, ArH); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 156.6, 156.3, 152.3, 140.8, 138.4, 137.4, 137.2, 136.4, 134.4, 132.0, 131.9, 129.9, 125.7, 125.3, 121.6, 121.3, 120.0, 118.0, 116.2, 116.0; *m/z* (ESP) 387 ($[M+H]^+$), found 387.1733, $C_{22}H_{20}N_6F$ requires 387.1714.

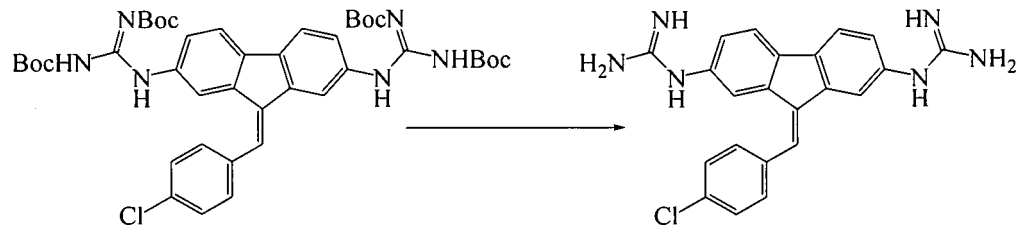
9-(4-chlorobenzylidene)-2,7-dinitro-9*H*-fluorene (85) To a suspension of 2,7-dinitro-*9H*-fluorene (1.00 g, 3.90 mmol) and KF:Al_2O_3 (0.65 g, 4.68 mmol) in anhydrous DMF (16 mL) was added *p*-chlorobenzaldehyde (0.60 g, 4.29 mmol), and the mixture was stirred overnight at room temperature. The resulting mixture was filtered under reduced pressure, washed with an excess of DCM and reduced *in vacuo* furnishing **85** as a flocculent yellow solid (1.23 g, 83%). Mp >252 °C (decomp); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.73 (d, 1H, J = 2.1 Hz, ArH), 8.58 (d, 1H, J = 2.1 Hz, ArH), 8.37 (dd, 1H, J = 1.9, 8.4 Hz, ArH), 8.32 (dd, 1H, J = 2.1, 8.4 Hz, ArH), 7.97 (m, 3H, ArH, CH), 7.55 (m, 4H, ArH); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 148.9, 148.7, 142.3, 137.0, 134.4, 131.8, 131.0, 129.8, 126.6, 122.8, 121.9, 119.8, 118.6, 114.5; anal. Found C 63.45, H 3.01, N 7.45, $C_{20}H_{11}N_2O_4Cl$ requires C 63.42, H 2.93, N 7.40.



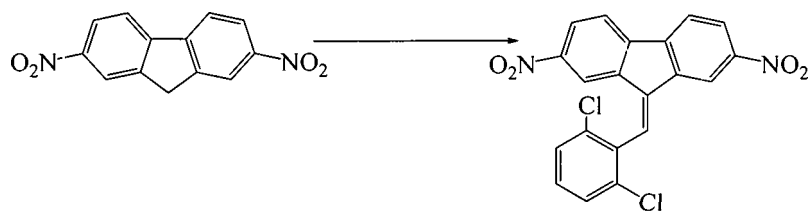
9-(4-chlorobenzylidene)-9*H*-fluorene-2,7-diamine (91) To a suspension of compound **85** (0.13 g, 0.34 mmol) and zinc (0.73 g, 11.14 mmol) in EtOH (4 mL) was added calcium chloride (24 mg, 0.22 mmol) in water (40 μL). The mixture was stirred for 2 hrs at reflux and filtered hot under reduced pressure, washed with excess hot EtOH and the filtrate reduced. Purification by column chromatography eluting with DCM:MeOH (8:2) afforded compound **91** as a red solid (88 mg, 81%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.60 (d, 2H, J = 8.0 Hz, ArH), 7.52 (d, 2H, J = 8.2 Hz, ArH), 7.45 (s, 1H, CH), 7.30 (m, 4H, ArH), 6.61 (m, 2H, ArH), 3.60 (brs, 4H, NH_2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 148.3, 147.1, 144.1, 141.0, 134.9, 129.7, 120.1, 119.8, 114.6, 111.1.



***Tert*-butyl(9-(4-chlorobenzylidene)-9*H*-fluorene-2,7-diyl)*bis*(azanediyl)*bis*((*tert*-butoxycarbonylamino)methan-1-yl-1-ylidene)dicarbamate (97)** Compound **91** (0.13 g, 0.41 mmol), triethylamine (0.34 mL, 2.46 mmol), 1,3-*bis*(*tert*-butoxycarbonyl)-2-methyl-thiopseudourea (0.25 g, 0.86 mmol), and mercury (II) chloride (0.26 g, 0.95 mmol) were suspended in anhyd. DMF (2.56 mL) and stirred overnight under a nitrogen atmosphere. The mixture was diluted with DCM, washed with Na_2SO_3 and filtered through a pad of celite. The filtrate was washed with water, brine, dried over Na_2SO_4, filtered and reduced *in vacuo*. The solid was recrystallised from DCM/MeOH after which purification by column chromatography eluting with DCM afforded compound **97** as a pale yellow solid (0.22 g, 67%). Mp >300°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 11.68 (s, 1H, NH), 11.64 (s, 1H, NH), 10.45 (s, 1H, NH), 10.15 (s, 1H, NH), 8.08 (s, 1H, CH), 7.79 (d, 1H, J = 1.7 Hz, ArH), 7.58 (m, 6H, ArH), 7.47 (d, 2H, J = 8.6 Hz, ArH), 7.36 (dd, 1H, J = 1.9, 8.2 Hz, ArH), 1.52 (m, 36H, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 164.1, 154.4, 153.8, 140.9, 138.7, 137.2, 136.6, 136.3, 135.9, 135.1, 135.0, 134.4, 131.3, 129.9, 129.1, 127.1, 124.0, 122.8, 120.6, 120.1, 120.0, 114.6, 28.6, 28.5; ν_{max} (Neat) / cm^{-1} 3305, (N-H), 2971 (Ar-H), 1718 (C=O), 1631 (N-H), 1367 (C-H), 1149 (C-O-CH_3), 740 (C-Cl); *m/z* (ESP) 803/805/807 ([M+H]$^+$); anal. Found C 62.72, H 6.51, N 10.39, $C_{42}H_{51}ClN_6O_8$ requires C 62.79, H 6.40, N 10.46.

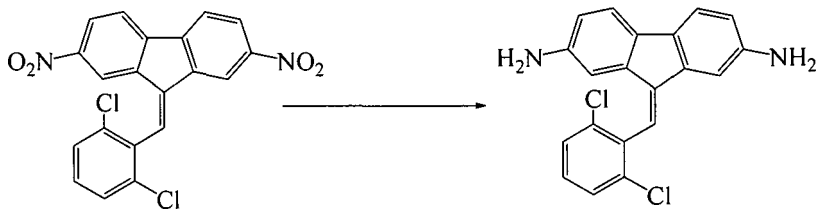


1,1'-(9-(4-chlorobenzylidene)-*9H*-fluorene-2,7-diyl)diguanidine (103) Compound **97** (0.10 g, 0.13 mmol) was suspended in a mixture of dry DCM (1.17 mL) and EtOH (0.78 mL), cooled to 0°C and saturated with gaseous hydrogen chloride. The mixture was sealed and stirred at room temperature for 4 days after which the mixture was reduced *in vacuo* and the residue crystallised with EtOH/Et_2O yielding compound **103** as a yellow solid (24 mg, 37%). Mp >300°C; ^{1}H NMR (DMSO, 400 MHz) δ 7.99 (s, 1H, CH), 7.96 (d, 1H, *J* = 7.8 Hz, ArH), 7.94 (d, 1H, *J* = 7.8 Hz, ArH), 7.86 (d, 1H, *J* = 1.7 Hz, ArH), 7.72 (d, 2H, *J* = 8.4 Hz, ArH), 7.53 (m, 10H, NH, ArH), 7.29 – 7.25 (dd, 1H, *J* = 1.9, 17.3 Hz, ArH), 7.27 (s, 1H, ArH); ^{13}C NMR (DMSO, 100 MHz) δ 156.6, 140.8, 137.1, 136.4, 135.0, 134.8, 134.5, 131.5, 129.1, 125.9, 121.7, 121.3; *m/z* (ESP) 403/405/407 ($[M+H]^+$), found 403.1438, $C_{22}H_{20}N_6{}^{35}Cl$ requires 403.1453.

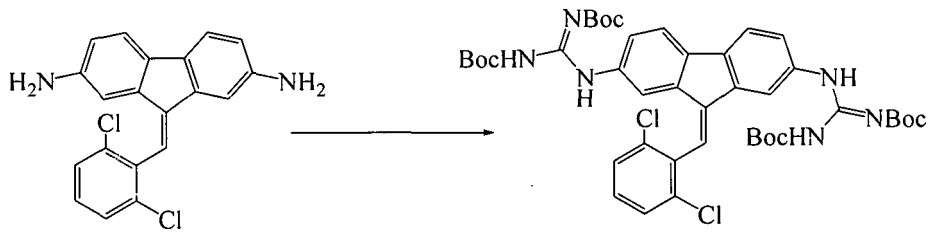


9-(2,6-dichlorobenzylidene)-2,7-dinitro-9*H*-fluorene (86) To a suspension of 2,7-dinitro-*9H*-fluorene (1.00 g, 3.90 mmol) and KF:Al_2O_3 (0.65 g, 4.68 mmol) in anhyd. DMF (16 mL) was added 2,6-dichlorobenzaldehyde (0.75 g, 4.29 mmol), after which the mixture was stirred overnight at room temperature. The resulting suspension was filtered under reduced pressure, washed with an excess of DCM and reduced *in vacuo* furnishing **86** as flocculent yellow solid (1.26 g, 78%). Mp 250°C (decomp); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.79 (d, 1H, *J* = 1.9 Hz, ArH), 8.39 (dd, 1H, *J* = 2.1, 8.4 Hz, ArH), 8.31 (dd, 1H, *J* = 1.9, 8.4 Hz, ArH), 7.96 (d, 1H, *J* = 8.5 Hz, ArH), 7.93 (d, 1H, *J* = 8.5 Hz, ArH), 7.73 (m, 2H, ArH), 7.56 (d, 1H, *J* = 1.1 Hz, ArH),

7.54 (s, 1H, CH), 7.46 (m, 1H, ArH); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 148.9, 148.7, 148.3, 144.3, 143.0, 140.8, 138.7, 136.9, 135.0, 132.8, 131.3, 129.1, 126.4, 125.2, 125.0, 121.8, 121.6, 120.1, 117.1; anal. Found C 58.22, H 2.11, N 6.33, $C_{20}H_{10}N_2O_4Cl_2$ requires C 58.13, H 2.44, N 6.78.

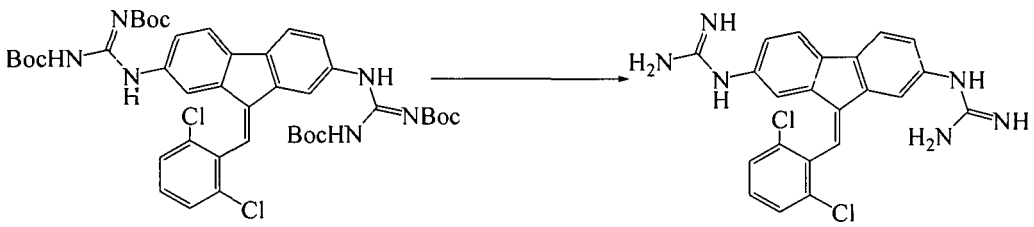


9-(2,6-dichlorobenzylidene)-9*H*-fluorene-2,7-diamine (92) To a suspension of compound **86** (0.15 g, 0.36 mmol) and zinc (0.77 g, 11.79 mmol) in EtOH (5 mL) was added calcium chloride (26 mg, 0.23 mmol) in water (40 μL). The mixture was stirred for 2 hrs at reflux and filtered hot under reduced pressure, washed with excess hot EtOH and the filtrate reduced. Purification by column chromatography eluting with DCM:MeOH (8:2) afforded compound **92** as a red solid (96 mg, 75%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.42 (d, 2H, J = 8.0 Hz, ArH), 7.30 (m, 3H, ArH), 7.15 (s, 1H, CH), 7.10 (d, 1H, J = 2.1 Hz, ArH), 6.65 (dd, 1H, J = 2.1, 8.0 Hz, ArH), 6.58 (dd, 1H, J = 2.1, 8.0 Hz, ArH), 6.06 (d, 1H, J = 2.1 Hz, ArH); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 145.2, 135.6, 129.6, 128.5, 126.5, 119.7, 119.5, 119.2, 116.3, 116.2, 111.8, 108.2; anal. Found C 67.38, H 4.30, N 7.19, $C_{20}H_{14}Cl_2N_2$ requires C 68.00, H 3.99, N 7.93.



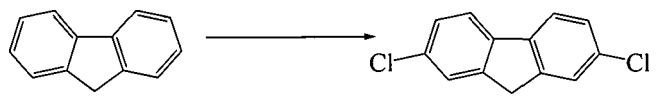
^{tert}-butyl(9-(2,6-dichlorobenzylidene)-9*H*-fluorene-2,7-diyl)*bis*(azanediyl)*bis*((^{tert}-butoxy-carbonylamino)methan-1-yl-1-ylidene)dicarbamate (98) Compound **92** (0.24 g, 0.70 mmol), triethylamine (0.59 mL, 4.21 mmol), 1,3-*bis*(*tert*-butoxycarbonyl)-2-methylthiopseudourea (0.43 g, 1.47 mmol), and mercury (II) chloride (0.44 g, 1.61 mmol) were suspended in anhyd. DMF

(4.22 mL) and stirred overnight under a nitrogen atmosphere. The mixture was diluted with DCM (150 mL), washed with Na_2SO_3 and filtered through a pad of celite. The filtrate was washed with water, brine, dried over Na_2SO_4, filtered and reduced *in vacuo*. The solid was recrystallised from DCM/MeOH after which purification by column chromatography eluting with DCM afforded the compound **98** as a yellow solid (0.39 g, 67%). Mp >342°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 11.64 (s, 1H, NH), 11.53 (s, 1H, NH), 10.43 (s, 1H, NH), 10.02 (s, 1H, NH), 8.07 (s, 1H, ArH), 7.63 (dd, 1H, J = 1.7, 8.2 Hz, ArH), 7.56 (m, 3H, ArH), 7.45 (d, 2H, J = 8.2 Hz, ArH), 7.34 (s, 1H, CH), 7.28 (d, 1H, J = 8.3 Hz, ArH), 6.68 (s, 1H, ArH), 1.51 (m, 36H, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 164.0, 153.9, 139.7, 139.4, 138.3, 137.7, 136.6, 136.3, 135.5, 134.7, 129.7, 128.6, 124.4, 123.5, 121.1, 120.1, 120.0, 119.6, 115.4, 83.9, 79.9, 28.6, 28.5; ν_{max} (Neat) / cm^{-1} 3305 (N-H), 3014 (Ar-H), 1720 (C=O), 1635 (C=N-H), 1369 (C-H), 1151 (C-O-CH_3); *m/z* (ESP) 837/839/841 ($[M+H]^+$), found 839.3116, $C_{42}H_{51}N_6O_8{}^{35}Cl_2$ requires 839.3125; anal. Found C 59.60, H 5.70, N 9.22, $C_{42}H_{50}N_6O_8Cl_2$ requires C 60.21, H 6.02, N 10.03.

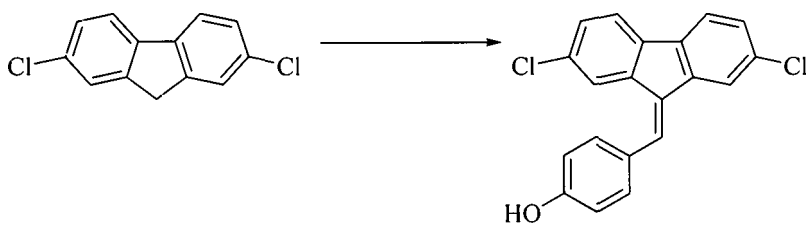


1,1'-(9-(2,6-dichlorobenzylidene)-9*H*-fluorene-2,7-diyl)diguanidine (104) Compound **98** (0.21 g, 0.25 mmol) was suspended in a mixture of anhyd. DCM (2.36 mL) and anhyd. EtOH (1.60 mL), cooled to 0°C and saturated with gaseous hydrogen chloride. The mixture was sealed and stirred at room temperature for 4 days after which the mixture was reduced *in vacuo* and the residue crystallised with EtOH/Et_2O yielding compound **104** as a yellow solid (98 mg, 70%). Mp >320°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.95 (d, 3H, J = 7.6 Hz, ArH), 7.80 (s, 1H, CH), 7.68 (d, 2H, J = 7.8 Hz, ArH), 7.58 (m, 5H, NH, ArH), 7.44 (brs, 4, NH), 7.33 (dd, 1H, J = 1.7, 8.2 Hz, ArH), 7.28 (dd, 1H, J = 1.9, 8.2 Hz, ArH), 6.50 (d, 1H, J = 1.9 Hz, ArH); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 156.7, 156.4, 139.7, 138.5, 137.9, 137.5, 137.1, 135.1, 134.9, 134.0, 133.6, 131.4, 129.1, 126.7, 126.3, 123.6, 121.7, 121.5, 120.1, 118.8; ν_{max} (Neat) / cm^{-1} 3635 (N-H), 1667 (C=N-H),

777 (C-Cl); *m/z* (ESP) 437/439/441 ($[M+H]^+$), found 437.1046, $C_{22}H_{19}N_6{}^{35}Cl_2$ requires 437.1048.

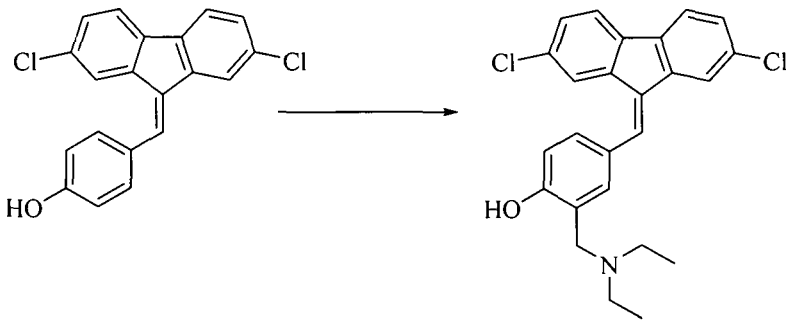


2,7-dichloro-*9H*-fluorene (110) *9H*-fluorene (3.00 g, 18.05 mmol) was added to *N*-chlorosuccinamide (2.29 g, 43.31 mmol) in MeCN (7.0 mL) and conc. HCl (1.45 mL) was added dropwise with caution. The mixture was stirred at room temperature for 1 day after which the solid was filtered and dried under vacuum. Recrystallisation from MeOH gave the desired compound **110** as white crystals (3.40 g, 86%). ^1H ($CDCl_3$, 400 MHz) δ 7.59 (d, 2H, J = 8.0 Hz, ArH), 7.63 (s, 2H, ArH), 7.48 (d, 2H, J = 8.0 Hz, ArH), 3.82 (s, 2H, CH_2), ^{13}C NMR ($CDCl_3$, 100 MHz) δ 144.3, 143.9, 133.0, 128.4, 126, 36.5; ν_{max} (Neat) / cm^{-1} 3298 (Ar-H), 2931 (C-H), 1606 (Ar), 1501 (Ar), 727 (C-Cl).



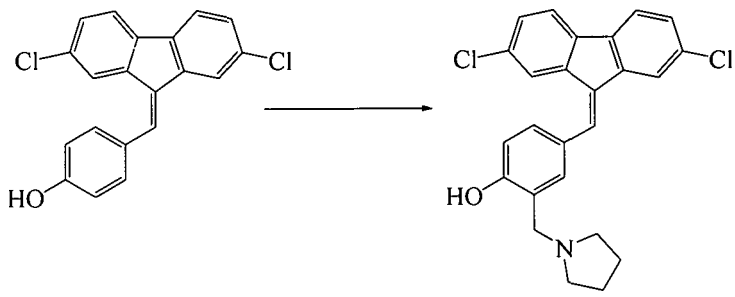
4-((2,7-dichloro-9*H*-fluoren-9-ylidene)methyl)phenol (111) 4-hydroxybenzaldehyde (0.52 g, 4.26 mmol) was added to a mixture of 2,7-dichlorofluorene (1.00 g, 4.26 mmol) and KF:Al_2O_3 (0.37 g, 4.26 mmol) in anhyd. DMF (10 mL) and heated to 150 °C overnight. The mixture was poured into water and extracted with EtOAc (2 x 100 mL). The organic extract was washed with water (2 x 50 mL), brine (50 mL) and dried over $MgSO_4$. Removal of solvent gave a yellow oil which was purified by column chromatography eluting with EtOAc: hexane (1:9) followed by recrystallisation from $CHCl_3$: hexane (1:1) giving the compound **111** as yellow crystals (1.08 g, 75%); Mp 106-108°C; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.69 (d, 1H, J = 1.5 Hz, ArH), 7.67 (d, 1H, J = 1.9 Hz, ArH), 7.62 (s, 1H, CH), 7.57 (d, 2H, J = 8.0 Hz, ArH), 7.47 (d, 2H, J = 8.0 Hz, ArH), 7.42 (ddd, 2H, J = 1.7, 8.2, 16.5 Hz, ArH), 6.94 (d, 2H, J = 8.7 Hz, ArH); ^{13}C NMR

($CDCl_3$, 100 MHz) δ 156.6, 141.6, 138.9, 138.3, 136.7, 133.9, 133.4, 132.9, 131.5, 130.2, 128.8, 128.5, 128.4, 124.6, 120.9, 116.1; ν_{max} (Neat) / cm^{-1} 3270 (O-H), 3083 (Ar-H), 2940 (C-H), 1592 (Ar), 1609 (Ar); *m/z* (ESP) 377/339/341 ([M-H]$^-$), found 337.0187, $C_{20}H_{11}O^{35}Cl_2$ requires 337.0180.



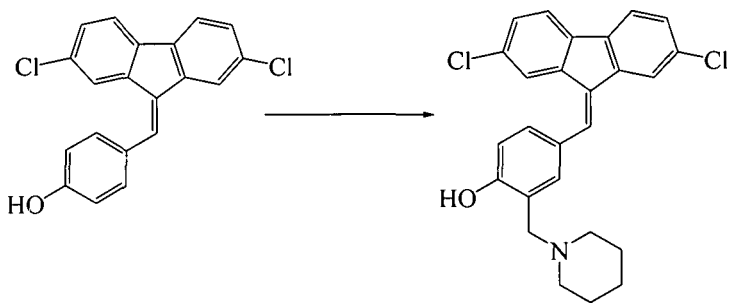
4-((2,7-dichloro-9*H*-fluoren-9-ylidene)methyl)-2-((diethylamino)methyl)phenol (112)

Formaldehyde (58 μL, 0.73 mmol) and diethylamine (264 μL, 2.56 mmol) were added to a solution of compound **111** (250 mg, 0.73 mmol) in $CHCl_3$ (4 mL) and heated to reflux for 2 hr. On cooling the solvent was removed *in vacuo* and the residue taken up in EtOAc (50 mL). The organic extract was washed with water (50 mL), brine (50 mL) before drying over $MgSO_4$. Purification by column chromatography eluting with MeOH: DCM (1:99) giving compound **112** as yellow crystals (127 mg, 41%). Mp 103°C; ^{1}H NMR($CDCl_3$, 400 MHz) δ 7.76 (d, 1H, *J* = 1.7 Hz, ArH), 7.70 (d, 1H, *J* = 1.7 Hz, ArH), 7.62 (s, 1H, CH), 7.58, (d, 2H, *J* = 8.0 Hz, ArH), 7.38 (dd, 1H, *J* = 1.9, 8.3 Hz, ArH), 7.29 (m, 3H, ArH), 6.91 (d, 1H, *J* = 8.2 Hz, ArH), 3.82 (s, 2H, CH_2), 2.70 (q, 4H, *J* = 7.2 Hz, CH_2), 1.16 (t, 6H, *J* = 7.2 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 159.9, 141.7, 138.8, 138.5, 136.5, 133.4, 133.1, 132.8, 131.1, 130.5, 130.2, 128.5, 128.1, 126.4, 124.5, 122.8, 120.9, 120.8, 116.9, 57.2, 46.8, 11.6; ν_{max} (Neat) / cm^{-1} 3554 (O-H), 3048 (Ar-H), 2938 (C-H), 1592 (Ar), 1494 (Ar), 1253 (C-N); *m/z* (ESP) 424/426/428 ([M+H]$^+$), found 424.1238, $C_{25}H_{24}NO^{35}Cl_2$; requires 424.1235; anal. Found C 70.27, H 5.45, N 3.22, $C_{25}H_{23}NOCl_2$ requires C 70.70, H 5.46, N 3.30.



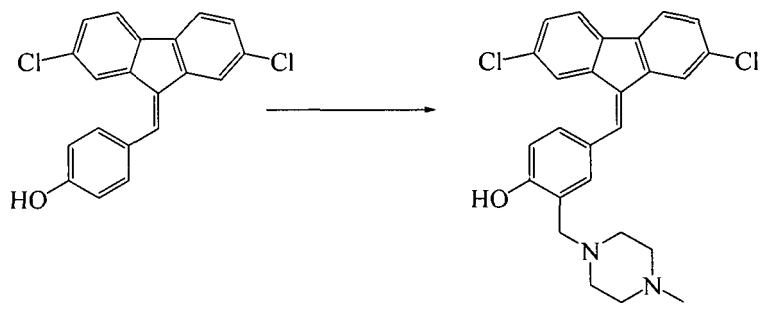
4-((2,7-dichloro-9*H*-fluoren-9-ylidene)methyl)-2-(pyrrolidin-1-ylmethyl)phenol (113)

Formaldehyde (58 μL, 0.73 mmol) and pyrolidine (214 μL, 2.56 mmol) were added to a solution of compound **111** (0.25 g, 0.73 mmol) in $CHCl_3$ (4.0 mL) and heated to reflux overnight. On cooling the solvent was removed *in vacuo* and the residue taken up into $CHCl_3$ (50 mL). The organic extract was washed with water (50 mL), brine (50 mL) and dried (Na_2SO_4). Purification by column chromatography eluting with MeOH: DCM (1:99) gave the title compound **113** as yellow crystals (0.19 g, 62%). Mp 182-183°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.77 (d, 1H, J = 1.9 Hz, ArH), 7.70 (d, 1H, J = 1.9 Hz, ArH), 7.62 (s, 1H, CH), 7.58 (d, 2H, J = 8.0 Hz, ArH), 7.39 (dd, 1H, J = 1.9, 8.4 Hz, ArH), 7.29 (m, 3H, ArH), 6.93 (d, 1H, J = 8.1 Hz, ArH), 3.87 (s, 2H, CH_2), 2.71 (bs, 4H, CH_2), 1.89 (s, 4H, CH_2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 160.9, 143.1, 140.2, 139.8, 137.9, 134.7, 134.6, 134.1, 132.3, 132.0, 131.2, 129.9, 129.5, 127.8, 125.9, 124.4, 122.3, 122.2, 122.1, 118.3, 60.3, 55.3, 25.4; *m/z* (ESP) 422/424/426 ($[M+H]^+$), found 422.1084, $C_{25}H_{22}N^{35}Cl_2O$ requires 422.1078; anal. Found C 68.97, H 4.92, N 3.17, $C_{25}H_{22}NOCl_2$ requires C 71.09, H 5.01, N 3.32.

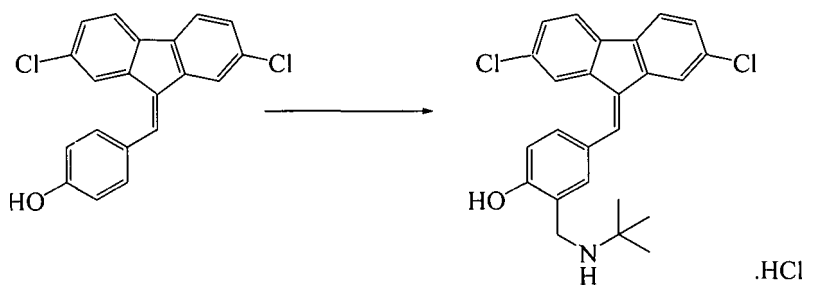


4-((2,7-dichloro-9*H*-fluoren-9-ylidene)methyl)-2-(piperidin-1-ylmethyl)phenol (114)

Formaldehyde (58 μL, 0.73 mmol) and piperidine (253 μL, 2.56 mmol) were added to a solution of compound **111** (0.25 g, 0.73 mmol) in $CHCl_3$ (5.0 mL). The resulting suspension was heated to 65 °C for 6 hr before a second portion of formaldehyde (58 μL, 0.73 mmol) was added and the solution left stirring at 65 °C overnight. On cooling, the solvent was removed under reduced pressure and the residue taken up into $CHCl_3$ (50 mL). The organic extract was washed with water (50 mL), brine (50 mL) and dried (Na_2SO_4). Removal of solvent gave a yellow solid which was purified by column chromatography eluting with MeOH: DCM (4:96) to give the title compound **114** as yellow crystals (212 mg, 67%). Mp 172°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.75 (d, 1H, J = 1.7 Hz, ArH), 7.70 (d, 1H, J = 1.7 ArH), 7.62 (s, 1H, CH), 7.58 (d, 2H, J = 8.2 Hz, ArH), 7.38 (dd, 1H, J = 1.9, 8.4 Hz, ArH), 7.29 (m, 3H, ArH), 6.92 (d, 1H, J = 8.4 Hz, ArH), 3 71 (s, 2H, CH_2), 2.57 (bs, 4H, CH_2), 1.68 (t, 4H, J = 5.3 Hz, CH_2) 1.53 (bs, 2H, CH_2); ^{13}C ($CDCl_3$, 100MHz) δ 159.6, 141.7, 138.8, 138.4, 136.5, 133.3, 133.1, 132.7, 131.0, 130.5, 130.4, 128.5, 128.2, 126.5, 124.5, 122.3, 121.0, 120.9, 120.8, 116.8, 62.3, 54.3, 26.2, 24.3; *m/z* (ESP) 436/438/440 ($[M+H]^+$), found 436.1234, $C_{26}H_{24}NO^{35}Cl_2$ requires 436.1235; anal. Found C 71.54, H 5.38, N 3.16, $C_{26}H_{23}NOCl_2$ requires C 71.60, H 5.31, N 3.21.

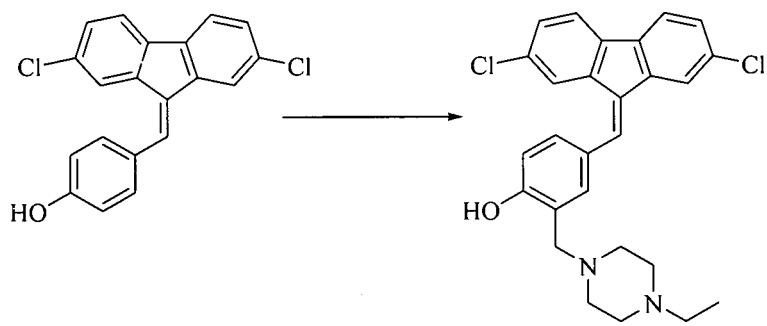


4-((2,7-dichloro-9*H*-fluoren-9-ylidene) methyl)-2-((4-methylpiperazin-1-yl) methyl) phenol (115) Formaldehyde (115 µL, 1.46 mmol) and *N*-methyl-piperazine (284 µL, 2.56 mmol) were added to a solution of compound **111** (0.25 g, 0.73 mmol) in $CHCl_3$ (5.0 mL) and heated to 65 °C overnight (16 hr).The reaction mixture was poured into water and the organic layer was washed with water (50 mL), brine (50 mL) and dried (Na_2SO_4). Removal of solvent gave a yellow oil, which was crystallized from EtOH and filtered to give the title compound **115** as yellow crystals (0.21 g, 64%). Mp 129-130°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.70 (dd, 2H, J = 1.7, 12.3 Hz, ArH), 7.59 (s, 1H, CH), 7.56 (d, 2H, J = 8.2 Hz, ArH), 7.37 (dd, 1H, J = 1.9, 8.2 Hz, ArH), 7.29 (m, 3H, ArH), 6.93 (d, 1H, J = 8.4 Hz, ArH), 3.76 (s, 2H, CH_2), 2.58 (brs, 8H, CH_2), 2.32 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 159.2, 141.6, 138.8, 138.4, 136.6, 133.4, 132.8, 130.8, 130.7, 130.5, 128.6, 128.2, 126.8, 124.5, 121.8, 120.9, 120.8, 116.9, 61.5, 55.3, 53.0, 46.2; *m/z* (ESP) 451/453/455 ($[M+H]^+$), found 451.1355, $C_{26}H_{25}N_2O^{35}Cl_2$ requires 451.1344; anal. Found C 68.80, H 5.33, N 6.16, $C_{26}H_{24}N_2OCl_2$ requires C 69.20, H 5.36, N 6.20.



2-((*tert*-butylamino)methyl)-4-((2,7-dichloro-9*H*-fluoren-9-ylidene)methyl)phenolhydrochloride (116) Formaldehyde (87 µL, 1.10 mmol) and *tert*-butylamine (269 µL, 2.56 mmol) were added to a solution of compound **111** (0.25 g, 0.73 mmol) in $CHCl_3$ (5.0 mL) and heated to reflux overnight. On cooling, the solvent was removed under reduced pressure and the residue

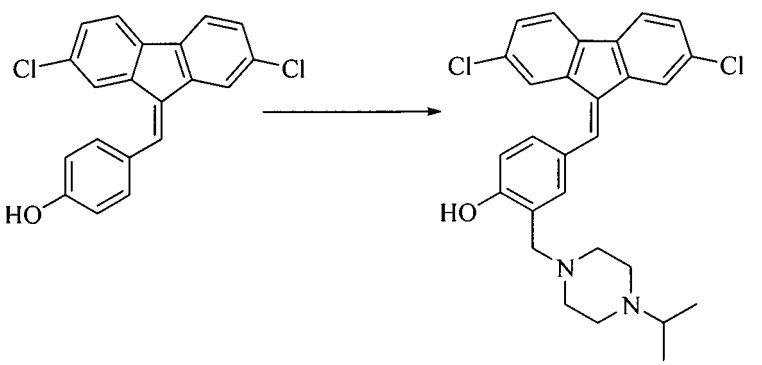
dissolved in DCM (50 mL). The organic extract was washed with water (50 mL), brine (50 mL), dried (Na_2SO_4) and reduced *in vacuo* yielding a yellow oil. Purification by crystallisation (EtOH) followed by addition of *iso*-propyl alcohol (20% HCl) gave the title compound **116** as a pale yellow solid (0.11 g, 33%). Mp 259-260°C, ^{1}H NMR (DMSO, 400 MHz) δ 8.10 (d, 1H, J = 1.7 Hz, ArH), 8.01 (s, 1H, CH), 7.94 (d, 1H, J = 8.2 Hz, ArH), 7.92 (d, 1H, J = 8.3 Hz, ArH), 7.74 (s, 1H, ArH), 7.67 (d, 1H, J = 1.9 Hz, ArH), 7.58 (dd, 1H, J = 1.7, 8.4 Hz, ArH), 7.45 (d, 2H, J = 8.2 Hz, ArH), 7.18 (d, 1H, J = 8.4 Hz, ArH), 4.08 (s, 2H, CH_2), 1.39 (s, 9H, CH_3); ^{13}C NMR (DMSO, 100 MHz) δ 158.9, 143.0, 140.5, 139.6, 138.1, 135.5, 134.8, 134.3, 134.0, 133.9, 131.5, 130.1, 129.7, 129.2, 125.3, 122.6, 122.4, 122.1, 120.8, 117.1, 59.1, 42.7, 26.3; *m/z* (ESP) 424/426/428 ($[M+H]^+$), found 424.1233, $C_{25}H_{24}NO^{35}Cl_2$ requires 424.1235; anal. Found C 64.80, H 5.28, N 2.88, $C_{25}H_{24}NOCl_3$ requires C 65.2, H 5.24, N 3.04.



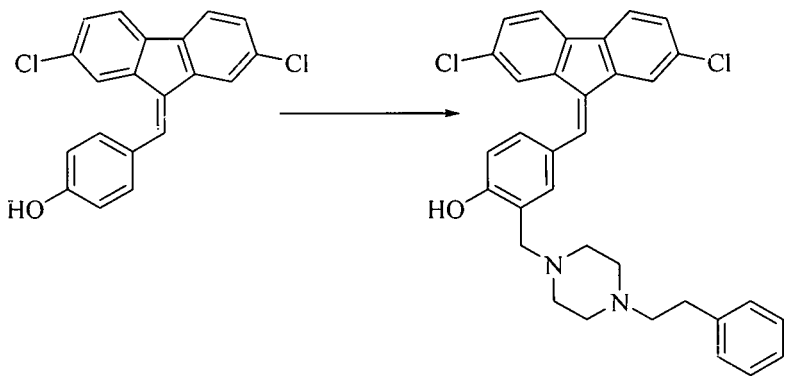
4-(2,7-dichloro-fluoren-9-ylidenemethyl)-2-(4-ethyl-piperazin-1-ylmethyl)-phenol (117)

Formaldehyde (58 μL, 0.73 mmol) and 1-ethyl-piperazine (330 μL, 2.58 mmol) were added to a solution of compound **111** (250 mg, 0.73 mmol) in $CHCl_3$ (4 mL). The resulting suspension was heated to 65 °C overnight. On cooling, the solvent was removed under reduced pressure and the residue taken up into $CHCl_3$ (50 mL). The organic extract was washed with water (50 mL), brine (50 mL) and dried over Na_2SO_4. Removal of solvent gave a yellow solid which was purified by column chromatography eluting with MeOH: DCM (4:96) to give the title compound **117** as yellow crystals (198 mg, 58%). Mp 134°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.72 (d, 1H, J = 1.9 Hz, ArH), 7.68 (d, 1H, J = 1.7 Hz, ArH), 7.60 (s, 1H, CH), 7.57 (d, 2H, J = 8.1 Hz, ArH), 7.39 (dd, 1H, J = 1.9, 8.4 Hz, ArH), 7.29 (m, 3H, ArH), 6.93 (d, 1H, J = 8.4 Hz, ArH), 3.76 (s, 2H, CH_2), 2.64 (brs, 7H, CH_2), 2.46 (q, 3H, J = 7.2 Hz, CH_2), 1.10 (t, 3H, J = 7.4 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 158.8, 141.3, 138.5, 138.4, 138.0, 136.2, 133.0, 132.4, 130.3, 130.1,

128.2, 127.9, 127.5, 126.4, 125.4, 124.1, 121.5, 120.7, 120.6, 120.5, 120.4, 116.5, 61.6, 61.5, 53.0, 52.9, 52.5, 49.4, 12.3; ν_{max} (Neat) / cm^{-1} 3562 (O-H), 3091 (Ar-H), 2942 (C-H), 1592 (Ar), 1496 (Ar), (C-N) 1253; *m/z* (ESP) 463/465/467 ([M-H]$^-$), found 463.1344, $C_{27}H_{26}N_2O^{35}Cl_2$ requires 463.1344; anal. Found C 69.29, H 5.69, N 5.92, requires C 69.68, H 5.63, N 6.02.

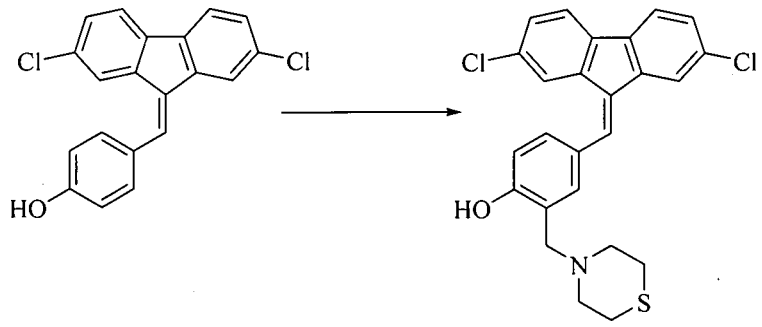


4-(2,7-dichloro-fluoren-9-ylidenemethyl)-2-(4-isopropyl-piperazin-1-ylmethyl)-phenol (118)
Formaldehyde (30 μL, 0.37mmol) and 1-isopropyl-piperazine (190 μL, 1.32 mmol) were added to a solution of compound **111** (128 mg, 0.37 mmol) in $CHCl_3$ (2.5 mL). The resulting suspension was heated to 65°C overnight. On cooling, the solvent was removed under reduced pressure and the residue taken up into $CHCl_3$ (30 mL). The organic extract was washed with water (30 mL), brine (30 mL) and dried over Na_2SO_4. Removal of solvent gave a yellow solid which was purified by column chromatography eluting with MeOH: DCM (4:96) to give the title compound **118** as yellow crystals (98 mg, 55%). Mp 135-138°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.73 (d, 1H, *J* = 1.9 Hz, ArH), 7.70 (d, 1H, *J* = 1.7 Hz, ArH), 7.62 (s, 1H, CH), 7.59 (d, 2H, *J* = 8.2 Hz, ArH), 7.39 (dd, 1H, *J* = 1.9, 8.4 Hz, ArH), 7.29 (m, 3H, ArH), 6.93 (d, 1H, *J* = 8.4 Hz, ArH), 3.76 (s, 2H, CH_2), 2.64 (brs, 8H, CH_2), 1.06 (d, 6H, *J* = 6.4 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 158.9, 141.3, 138.4, 138.1, 136.3, 133.0, 132.6, 132.5, 130.4, 130.1, 128.3, 127.9, 127.6, 125.4, 124.2, 121.6, 120.7, 120.6, 120.5, 116.6, 63.7, 63.5, 53.9, 53.5, 48.8, 19.4, 17.2; *m/z* (ESP) 477/479/481 ([M-H]$^-$), found 477.1500, $C_{28}H_{27}N_2O^{35}Cl_2$ requires 477.1505; anal. Found C 69.68, H 5.82, N 5.48, $C_{28}H_{28}N_2OCl_2$ requires C 70.14, H 5.88, N 5.84



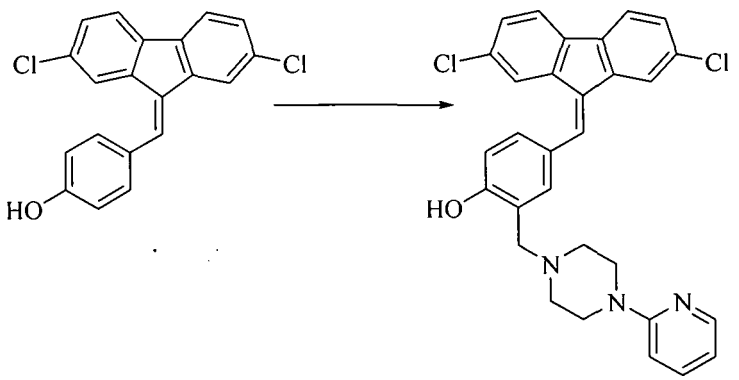
4-(2,7-dichloro-fluoren-9-ylidenemethyl)-2-(4-phenethyl-piperazin-1-ylmethyl)-phenol(119)

Formaldehyde (58 μL, 0.73 mmol) and 1-(2-phenylethyl) piperazine (0.4 mL, 2.06 mmol) were added to a solution of compound **111** (0.20 g, 0.58 mmol) in $CHCl_3$ (3.0 mL). The resulting suspension was heated to 65°C overnight. On cooling, the solvent was removed *in vacuo* and the residue taken up into $CHCl_3$ (30 mL). The organic extract was washed with water (30 mL), brine (30 mL) and dried (Na_2SO_4). Removal of solvent gave a yellow solid which was purified by column chromatography eluting with MeOH: DCM (4:96) to give the title compound **119** as yellow crystals (0.17 g, 54%). Mp 185-186°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.72 (d, 1H, J =1.9 Hz, ArH), 7.70 (d, 1H, J = 1.7 Hz, ArH), 7.62 (s, 1H, CH), 7.58 (d, 2H, J = 8.0 Hz, ArH), 7.56 (d, 2H, J = 8.0 Hz, ArH), 7.38 (dd, 1H, J = 1.9, 8.3 Hz, ArH), 7.29 (m, 3H, ArH), 7.20 (m, 4H, ArH), 3.77 (s, 2H, CH_2), 2.96 (d, 2H, J = 7.8 Hz, CH_2), 2.81 (m, 6H, CH_2), 2.64 (brt, 4H, J = 7.0 Hz, CH_2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 159.2, 138.9, 138.8, 138.4, 136.6, 133.4, 129.7, 129.5, 129.0, 128.8, 128.6, 128.2, 127.9, 125.7, 121.8, 121.4, 121.0, 116.9, 61.5, 60.5, 53.3, 52.9, 34.0; *m/z* (ESP) 539/541/543 ([M-H]$^-$), found 539.1657, $C_{33}H_{29}N_2O^{35}Cl_2$ requires 539.1672; anal. Found C 72.90, H 5.70, N 4.69, $C_{33}H_{30}N_2OCl_2$ requires C 73.19, H 5.58, N 5.17

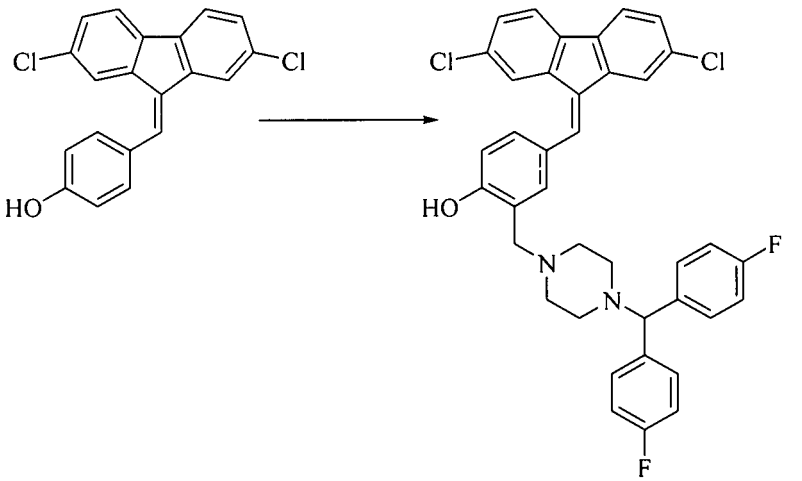


4-(2,7-dichloro-fluoren-9-ylidenemethyl)-2-thiomorpholin-4-ylmethyl-phenol (120)

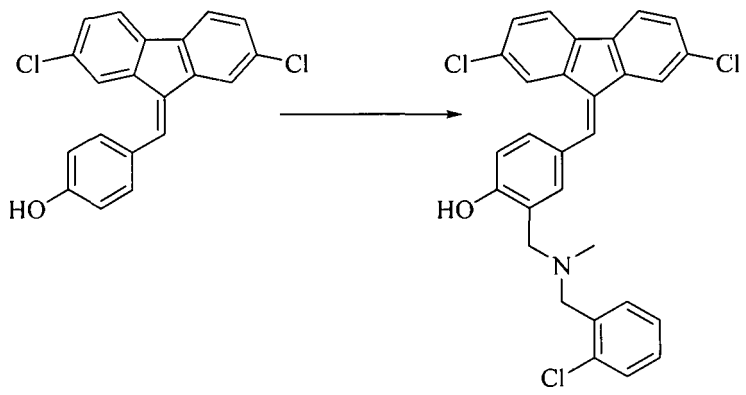
Formaldehyde (58 μL, 0.73 mmol) and thiomorpholine (250 μL, 2.56 mmol) were added to a solution of compound **111** (0.25 g, 0.73 mmol) in $CHCl_3$ (5.0 mL). The resulting suspension was heated to 65°C overnight. On cooling, the solvent was removed under reduced pressure and the residue taken up into $CHCl_3$ (50 mL). The organic extract was washed with water (50 mL), brine (50 mL) and dried (Na_2SO_4). Removal of solvent gave a yellow solid which was purified by column chromatography eluting with MeOH: DCM (4:96) to give the title compound **120** as yellow crystals (0.18 g, 54%). Mp 147°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.69 (d, 1H, J = 1.7 Hz, ArH), 7.68 (d, 1H, J = 1.9 Hz, ArH), 7.61 (s, 1H, CH), 7.58 (dd, 2H, J = 1.0, 8.2 Hz, ArH), 7.39 (dd, 1H, J = 1.9, 8.2 Hz, ArH), 7.29 (m, 3H, ArH), 6.94 (d, 1H, J = 8.3 Hz, ArH), 3.76 (s, 2H, CH_2), 2.91 (brs, 4H, CH_2), 2.78 (brs, 4H, CH_2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 159.0, 146.4, 141.6, 138.9, 138.4, 136.6, 133.6, 133.4, 132.7, 130.8, 130.7, 130.6, 128.6, 128.3, 127.0, 124.5, 121.5, 121.0, 120.9, 120.8, 117.0, 62.4, 54.9, 28.3; *m/z* (ESP) 454/456/458 ($[M+H]^+$), found 454.0799, $C_{25}H_{22}NOS^{35}Cl_2$ requires 454.0777; anal. Found C 66.89, H 4.51, N 2.58, $C_{25}H_{21}NOSCl_2$ requires C 66.08, H 4.66, N 3.08



4-(2,7-dichloro-fluoren-9-ylidenemethyl)-2-(4-pyridin-2-yl-piperazin-1-ylmethyl)-phenol (121) Formaldehyde (60 μL, 0.73 mmol) and 1-(2-pyridyl)piperazine (0.4 mL, 2.56 mmol) were added to a solution of compound **111** (0.25 g, 0.73 mmol) in $CHCl_3$ (5.0 mL). The resulting suspension was heated to 65°C overnight (20 hr). On cooling, the solvent was removed under reduced pressure and the residue taken up into $CHCl_3$ (50 mL). The organic extract was washed with water (50 mL), brine (50 mL) and dried (Na_2SO_4). Removal of solvent gave a yellow solid which was purified by column chromatography eluting with MeOH: DCM (4:96) to give the title compound **121** as yellow crystals (180mg, 48%). Mp 164°C; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.20 (dd, 1H, J = 1.7, 5.1 Hz, ArH), 7.71 (dd, 2H, J = 1.9, 5.9 Hz, ArH), 7.62 (s, 1H, CH), 7.59 (d, 2H, J = 8.2 Hz, ArH), 7.49 (m, 1H, ArH), 7.41 (dd, 1H, J = 1.9, 8.6 Hz, ArH), 7.30 (m, 3H, ArH), 6.97 (d, 1H, J = 8.2 Hz, ArH), 6.67 (m, 2H, ArH), 3.81 (s, 2H, CH_2), 3.66 (brs, 4H, CH_2), 2.76 (brs, 4H, CH_2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 159.6, 159.0, 148.4, 141.6, 138.9, 138.4, 137.9, 136.6, 133.5, 133.5, 132.8, 130.8, 130.6, 128.6, 128.3, 127.0, 125.7, 124.5, 121.7, 121.0, 120.9, 120.8, 117.0, 114.2, 107.5, 61.7, 52.8, 45.5; *m/z* (ESP) 512/514/516 ($[M+H]^+$), found 512.1296, $C_{30}H_{25}N_3O^{35}Cl_2$ requires 512.1302; anal. Found C 69.29, H 4.96, N 7.50, $C_{30}H_{24}N_3OCl_2$ requires C 70.04, H 4.90, N 8.17

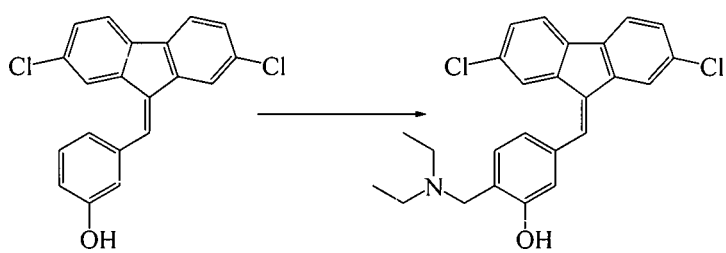


2-(4-(*Bis*-(4-fluoro-phenyl)-methyl)-piperazin-1-ylmethyl)-4-(2,7-dichloro-fluoren-9-ylidenemethyl)-phenol (122) Formaldehyde (23 μL, 0.29 mmol) and 1-*bis*-(4-fluorophenyl)-methyl piperazine (0.29 g, 1.0 mmol) were added to a solution of compound **111** (100 mg, 0.29 mmol) in $CHCl_3$ (3.0 mL). The resulting suspension was heated to 65°C overnight. On cooling, the solvent was removed *in vacuo* and the residue taken up into $CHCl_3$ (20 mL). The organic extract was washed with water (20 mL), brine (20 mL) and dried (Na_2SO_4). Removal of solvent gave a yellow solid which was purified by column chromatography eluting with MeOH: DCM (4:96) to give the title compound **122** as yellow crystals (85mg, 46%). Mp 249°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.69 (d, 1H, J = 1.7 Hz, ArH), 7.68 (d, 1H, J = 1.9 Hz, ArH), 7.61 (s, 1H, CH), 7.59 (d, 2H, J = 8.2 Hz, ArH), 7.32 (m, 8H, ArH), 6.98 (d, 2H, J = 8.7 Hz, ArH), 6.96 (d, 2H, J = 8.7 Hz, ArH), 6.90 (d, 1H, J = 8.4 Hz, ArH), 4.28 (s, 1H, CH), 3.75 (s, 2H, CH_2), 2.66 (brs, 4H, CH_2), 2.48 (brs, 4H, CH_2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 163.5, 159.1, 138.4, 138.1, 136.6, 133.4, 132.7, 130.7, 130.5, 129.6, 129.5, 128.6, 128.3, 126.8, 124.5, 121.8, 120.9, 120.8, 116.9, 115.9, 115.7, 74.5, 61.5, 53.2, 51.8; anal. Found C 70.92, H 4.74, N 4.45, $C_{38}H_{30}Cl_2F_2N_2$ requires C 71.36, H 4.73, and N 4.38.



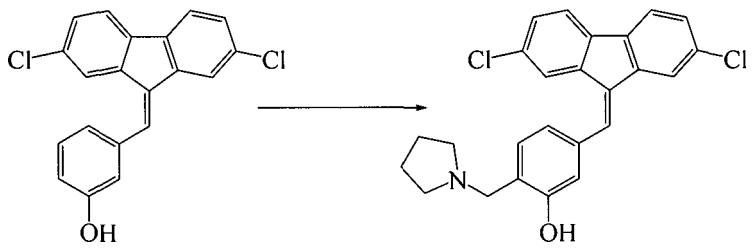
2-{((2-chloro-benzyl)-methyl-amino)-methyl}-4-(2, 7-dichloro-fluoren-9-ylidenemethyl)-phenol (123) Formaldehyde (58 μL, 0.73 mmol) and 2-chloro-*N*-methyl-benzylamine (360 μL, 2.56 mmol) were added to a solution of compound **111** (250 mg, 0.73 mmol) in $CHCl_3$ (5 mL). The resulting suspension was heated to 65°C overnight. On cooling, the solvent was removed under reduced pressure and the residue taken up into $CHCl_3$ (50 mL). The organic extract was washed with water (50 mL), brine (50 mL) and dried over Na_2SO_4. Removal of solvent gave a yellow solid which was purified by column chromatography eluting with MeOH: DCM (4:96) to give the title compound **123** as yellow crystals (196 mg, 53%). Mp 159°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.74 (d, 1H, J = 1.9 Hz, ArH), 7.70 (d, 1H, J = 1.9 Hz, ArH), 7.62 (s, 1H, CH), 7.58 (d, 2H, J = 8.1 Hz, ArH), 7.55 (d, 2H, J = 8.1 Hz, ArH), 7.41 (m, 4H, ArH), 7.29 (m, 3H, ArH), 6.94 (d, 1H, J = 8.4 Hz, ArH), 3.80 (s, 4H, CH_2), 2.35 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 156.8, 148.7, 138.8, 133.0, 132.0, 130.8, 130.4, 130.3, 129.7, 128.6, 128.2, 127.3, 125.7, 124.5, 122.5, 121.0, 120.9, 120.8, 116.9, 61.1, 60.9, 41.7, 41.5; *m/z* (ESP) 512/514/516 ($[M+H]^+$), found 506.0856, $C_{29}H_{23}NO^{35}Cl_3$ requires 506.0845; anal. Found C 68.38, H 4.52, N 2.38, $C_{29}H_{22}NOCl_3$ requires C 68.72, H 4.37, N 2.76.

3-((2,7-dichloro-9*H*-fluoren-9-ylidene)methyl)phenol (124) 3-hydroxybenzaldehyde (0.52 g, 4.26 mmol) was added to a mixture of 2,7-dichlorofluorene **110** (1.00 g, 4.26 mmol) and KF:Al_2O_3 (0.37 g, 4.26 mmol) in anhyd. DMF (10 mL) and heated to 150°C overnight. The mixture was poured into water and extracted with EtOAc (2 x 100 mL). The organic extract was washed with water (2 x 50 mL), brine (50 mL) and dried ($MgSO_4$). Removal of solvent gave a yellow oil which was purified by column chromatography eluting with EtOAc: hexane (1:9). Recrystallisation from $CHCl_3$: hexane (1:1) gave the title compound **124** as yellow crystals (0.87 g, 60%). ^{1}H ($CDCl_3$, 400 MHz) δ 7.71 (d, 1H, *J* = 1.7 Hz, ArH), 7.63 (s, 1H, CH), 7.57 (dd, 2H, *J* = 3.4, 8.2 Hz, ArH), 7.53 (d, 1H, *J* = 1.7 Hz, ArH), 7.35 (m, 2H, ArH), 7.28 (dd, 1H, *J* = 1.9, 8.2 Hz, ArH), 7.12 (d, 1H, *J* = 7.4 Hz, ArH), 6.99 (bs, 1H, OH), 6.91 (dd, 1H, *J* = 2.3, 8.2 Hz, ArH), ^{13}C NMR ($CDCl_3$, 100 MHz) δ 156.2, 137.7, 133.5, 133.0, 130.5, 129.6, 129.1, 128.8, 125.1, 122.0, 121.1, 120.9, 116.2; ν_{max} (Neat) / cm^{-1} 3272 (O-H), 3083 (Ar-H), 2940 (C-H), 1509 (Ar), 1619 (Ar); *m/z* (CI) 338 ([M-H]$^-$).



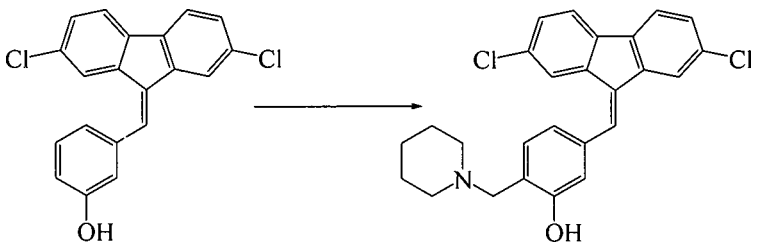
5-((2,7-dichloro-9*H*-fluoren-9-ylidene)methyl)-2-((diethylamino)methyl)phenol (125) Formaldehyde (58 μL, 0.73 mmol) and diethylamine (264 μL, 2.56 mmol) were added to a solution of compound **124** (0.25 g, 0.73 mmol) in $CHCl_3$ (4.0 mL) and heated to reflux for 1 hr. On cooling the solvent was removed under reduced pressure and the residue taken up in EtOAc (50 mL). The organic extract was washed with water (50 mL), brine (50 mL) before drying

($MgSO_4$). Purification by column chromatography eluting with MeOH: DCM (1:99) gave the title compound **125** as yellow crystals (0.17 g, 57%). Mp 112°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.71 (d, 1H, J = 1.7 Hz, ArH), 7.64 (s, 1H, CH), 7.57 (m, 3H, ArH), 7.32 (dd, 1H, J = 1.7, 8.2 Hz, ArH), 7.26 (dd, 1H, J = 1.7, 8.2, ArH), 7.06 (d, 1H, J = 8.2 Hz, ArH), 6.95 (d, 2H, J = 6.8 Hz, ArH), 3.85 (s, 2H, CH_2), 2.68 (q, 4H, J = 7.0, 14.2 Hz, CH_2), 1.16 (t, 6H, J = 7.2, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 159.1, 141.4, 138.9, 136.9, 136.5, 135.0, 133.4, 132.9, 130.5, 128.9, 128.8, 128.5, 125.3, 123.4, 121.1, 120.9, 120.8, 119.9, 116.9, 57.3, 46.9, 11.6; *m/z* (ESP) 422/424/426 ([M-H]$^-$), found 422.1063, $C_{25}H_{22}NO^{35}Cl_2$ requires 422.1078; anal. Found C 70.49, H 5.44, N 3.33, $C_{25}H_{23}NOCl_2$ requires C 70.70, H 5.46, N 3.30.



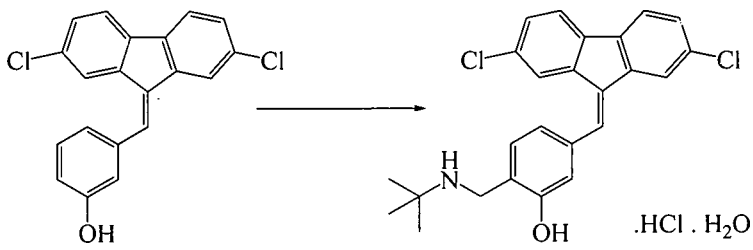
5-((2,7-dichloro-9*H*-fluoren-9-ylidene)methyl)-2-(pyrrolidin-1-ylmethyl)phenol (126)

Formaldehyde (39 μL, 0.49 mmol) and pyrolidine (129 μL, 1.54 mmol) were added to a solution of compound **124** (0.15 mg, 0.44 mmol) in $CHCl_3$ (4.0 mL) and heated to reflux overnight. On cooling, the solvent was removed under reduced pressure and the residue dissolved in $CHCl_3$ (30 mL). The organic layer was washed with water (50 mL), brine (50 mL) and dried (Na_2SO_4). Purification by column chromatography MeOH: DCM (1:99) gave the title compound **126** as yellow needles (0.13 mg, 73%). Mp 149-150°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.71 (d, 1H, J = 1.9 Hz, ArH), 7.65 (s, 1H, CH), 7.57 (m, 3H, ArH), 7.33 (dd, 1H, J = 1.7, 8.2 Hz, ArH), 7.26 (dd, 1H, J = 1.9, 8.2, ArH), 7.07 (d, 1H, J = 8.2 Hz, ArH), 6.96 (d, 2H, J = 6.2 Hz, ArH), 3.91 (s, 2H, CH_2), 2.71 (s, 4H, CH_2), 1.88 (s, 4H, CH_2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 158.9, 141.5, 138.9, 138.4, 136.9, 136.6, 134.9, 133.5, 133.0, 130.5, 128.9, 128.6, 125.3, 123.7, 121.1, 120.9, 120.8, 120.0, 116.8, 59.2, 53.9, 24.1; ν_{max} (Neat) / cm^{-1} 3585 (O-H), 3073 (Ar-H), 2938 (C-H), 1587 (Ar), 1496 (Ar), 1270 (C-N); *m/z* (ESP) 422/424/426 ([M+H]$^+$), found 422.1075, $C_{25}H_{22}NO^{35}Cl_2$ requires 422.1078; anal. Found C 70.84, H 5.02, N 3.28, $C_{25}H_{21}NOCl_2$ requires C 71.0, H 5.01, N 3.32.



5-((2,7-dichloro-9*H*-fluoren-9-ylidene)methyl)-2-(piperidin-1-ylmethyl) phenol (127)

Formaldehyde (39 μL, 0.49 mmol) and piperidine (152 μL, 1.54 mmol) were added to a solution of compound **124** (0.15 g, 0.44 mmol) in $CHCl_3$ (4.0 mL) and heated to reflux overnight. On cooling, the solvent was removed under reduced pressure and the residue dissolved in $CHCl_3$ (30 mL). The organic layer was washed with water (50 mL), brine (50 mL) and dried (Na_2SO_4). Purification by column chromatography eluting with MeOH: DCM (1:99) followed by recrystallisation from $CHCl_3$: EtOH (1:2) gave the title compound **127** as yellow crystals (0.14 g, 72%). Mp 171-172°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.70 (d, 1H, J = 1.9 Hz, ArH), 7.64 (s, 1H, CH), 7.56 (m, 3H, ArH), 7.31 (dd, 1H, J = 1.7, 8.2 Hz, ArH), 7.26 (dd, 1H, J = 1.9, 8.2, ArH), 7.05 (d, 1H, J = 8.0 Hz, ArH), 6.95 (m, 2H, ArH), 3.75 (s, 2H, CH_2), 2.57 (bs, 4H, CH_2), 1.67 (t, 4H, J = 5.3 Hz, CH_2), 1.52 (bs, 2H, CH_2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 158.9, 141.5, 138.9, 138.3, 136.9, 136.5, 134.8, 133.4, 132.9, 130.4, 129.1, 128.8, 128.6, 125.3, 122.8, 121.1, 120.9, 120.8, 120.0, 116.9, 62.4, 54.4, 26.3, 24.3; *m/z* (ESP) 436/438/440 ($[M+H]^+$), found 436.1229, $C_{26}H_{24}NO^{35}Cl_2$ requires 436.1235; anal. Found C 71.44, H 5.31, N 3.17, $C_{26}H_{23}NOCl_2$ requires C 71.60, H 5.31, N 3.21.



2-((*tert*-butylamino)methyl)-5-((2,7-dichloro-9*H*-fluoren-9-ylidene)methyl)-phenolhydrochloride (128) Formaldehyde (23 μL, 0.29 mmol) and *tert*-butylamine (106 μL, 1.0 mmol) were added to a solution of compound **124** (0.10 g, 0.29 mmol) in $CHCl_3$ (2.0 mL) and heated to reflux overnight. On cooling, the solvent was removed under reduced pressure and the residue dissolved in DCM (50 mL). The organic extract was washed with water (50 mL), brine (50 mL) and dried (Na_2SO_4). Removal of solvent gave a yellow oil which was purified by column chromatography MeOH: DCM (0.5:99.5) to give the title compound **128** as a yellow oil. The oil was treated with *iso*-propyl alcohol (20% HCl), stirred and the solid filtered, washed (EtOH) and dried *in vacuo* to give the title compound **128** as a pale yellow solid (82 mg, 61%). Mp 259-260°C, ^{1}H NMR (DMSO, 400 MHz) δ 8.78 (brs, 1H, NH), 8.14 (d, 1H, J = 1.8 Hz, ArH), 8.07 (s, 1H, CH), 7.94 (t, 2H, J = 8.3 Hz, ArH), 7.63 (d, 1H, J = 2.0 Hz, ArH), 7.61 (s, 1H, ArH), 7.48 (m, 2H, ArH), 7.21 (s, 1H, ArH), 7.12 (d, 1H, J = 7.7 Hz, ArH), 4.11 (s, 2H, CH_2), 1.39 (s, 9H, CH_3); ^{13}C NMR (DMSO, 100 MHz) δ 156.7, 141.1, 138.9, 137.9, 137.5, 136.3, 133.9, 132.8, 132.5, 131.9, 129.2, 128.7, 123.9, 122.4, 122 0, 121.6, 120.1, 119.9, 115.8, 57.2, 25.8, 25.5; *m/z* (ESP) 424/426/428 ($[M+H]^+$), found 424.1229, $C_{25}H_{24}NO^{35}Cl_2$ requires 424.1235; anal. Found C 63.85, H 5.38, N 2.53, $C_{25}H_{25}NOCl_3$ requires C 63.91, H 5.36, N 2.98.

5.2.1 Preparation of β-hematin

Hemin (0.4176 g) was dissolved in NaOH (0.1M, 83.5 mL) and HCl (1.0 M, 8.35 mL) introduced to give a red precipitate. The mixture was stirred for a few minutes and sodium acetate (pH 5.0) added. This was heated for 2hrs at 60°C, cooled on ice, filtered (8 μM Millipore nitrocellulose filter) under reduced pressure and washed with excess water until the filtrate ran clear. The brown crystals were dried over P_2O_5 for 3 days. ν_{max} (Nujol / cm^{-1}) 1660.3 (C=O), 1209.6 (C-O).

5.2.2 Determination of Inhibition of Hemozoin Formation

Using an eppendorf preparation 10 μL ghosts, 780 μL NaOAc (0.5 M, pH 5.2), 10 μL HCl (0.1 M), 100 μL drug (in ammonium acetate), and 100 μL hemin (3 mM in 0.1 M NaOH) were incubated at 37°C for 72 hours, after which the samples were centrifuged for 20 minutes yielding a small green pellet. The supernatant was carefully extracted and the pellet washed with 1 mL $NaHCO_3$ solution (0.23 M, 3% SDS, pH 9.1), centrifuged for 20min and the supernatant removed with caution. The washing/extraction procedure was repeated twice more after which 0.5ml NaOH (0.1 M) was introduced and the mixture left standing at room temperature for 1hr. The green solution (0.5 mL) was transferred to a 48-well plate and the absorbance obtained. This procedure was repeated for all drugs in triplicate at 8 concentrations over a concentration gradient.

5.2.3 Determination of Inhibition of Parasite Growth

In vitro antimalarial activity was measured using the [^{3}H]-hypoxanthine incorporation assay with the appropriate strains of *P. falciparum*.[152]

CHAPTER VI

Appendix

6.0 High-Throughput Assay

Due to the inconsistent values obtained across triplicate wells for the β-hematin inhibition assay, we further probed the system to ascertain the optimum conditions for β-hematin formation, as shown in Table 1. In addition to this, the concentration of both hemin and drug was varied in order to obtain the most favourable conditions for a sigmoidal IC_{50} curve. Furthermore, ghost erythrocytes were used in place of β-hematin to assess their viability in this test system.

Table 1. Inhibition of Hemozoin formation *in vitro*

Entry	Drug	Haemin (10 mM) / μL	β-Haematin / μL in NH_2OAc	Ghosts / μL
1	**CQ + Amidine Series**	1.25	2.5	0
2	**CQ + Amidine Series**	1.25	2.5 In DMSO	0
3	**CQ**	1.25	0	5
4	**CQ**	1.25	5	0
5	**0**	5.00	0-150	0
6	**0**	1.25	0-150	0
7	**CQ**	1.25	50	0
8	**0**	0	0-150	0
9	**0**	0-10	5	0
10	**0**	0	0	0-50

Parameters adjusted in order to obtain the optimal conditions for FP crystal growth and reproducibility of inhibition data for the various drugs tested in triplicate.

Table 1 shows the parameters adjusted in order to improve the assay system. Firstly we addressed the disproportionate quantities of β-hematin present in each of the drug concentration wells by dissolving β-hematin in DMSO, thereby ensuring that the concentration of β-hematin

present in each well was accurate and consistent. This assay was performed for amidines **6-18** and CQ, all of which gave questionable results. Control wells absent of the test drugs were found to contain very little β-hematin. We attribute this property to the use of DMSO; presumably DMSO is able to solvate the iron-carboxylate bonds forming the β-hematin dimer. This then leads to the formation of the monomer thus the crystal seed for β-hematin formation is no longer present or is present but at an inadequate concentration (entry 2, Table 5).

Ghost membranes are commonly used in the inhibition of hemozoin formation assay. However as previously mentioned, due to the arduous method associated with the Ghost assay we were keen to avoid it. However, it was clear at this point that we would have to assess the use of ghosts and therefore we attempted to combine the two methods by using ghosts as a substitute for β-hematin, in a 48 well plate as oppose to eppendorfs (commonly used for Ghost method, Figure 4) as shown in Table 5, entry 3. However inconsistent results were obtained, shown in subsequent tests to be due to insufficient agitation of the plate (achieved more efficiently with eppendorfs) in order to obtain the hemozoin pellet (hemozoin pellet obtained in order to quantify the amount of hemozoin present thereby determining the potency of the drug versus hemozoin formation).

We then considered that the problem may be due to both a varied and/or insufficient concentration of β-hematin. The reaction was therefore repeated using an increased concentration of β-hematin as depicted in table 5, entry 4 however we did not obtain consistent and robust data. We then attempted to vary the concentration of β-hematin while maintaining a hemin concentration double that previously used (entry 5), in addition to varying the concentration β-hematin while maintaining a fixed concentration of hemin (entry 6). This was attempted in order to ascertain the optimum hemin and β-hematin concentrations respectively. These results led to the conclusion that maintaining a constant concentration of β-hematin 20 times that previously used (this concentration gave the most promising results of the previous tests) would give acceptable levels of β-hematin formation across the triplicate wells. However, when the drugs were introduced the results again were not consistent across the concentration range in order to obtain the required sigmoid curve for the inhibition plot (see Figure 6) and thus we embarked on the last three experiments as entries 8, 9 and 10 were we; observed the uninhibited growth of β-

hematin (8), varied the concentration of hemin while keeping β-hematin concentration constant in order to ascertain if the concentration of hemin was the issue (9), and observed the uninhibited growth of ghosts to confirm that they are able to do so in this test method (10). The results of which led us believe that a high-throughput method with β-hematin would require further investigation which would be time consuming.

As one last attempt to improve this methodology we compared Eppendorf/Ghost and plate/Ghost methods in order to develop some form of high-throughput technique. We were keen to use 48-well plates as the quantification method since reading of the plate is much quicker than the alternative colorimetric techniques. As mentioned, ghosts are commonly used in Eppendorfs thus to assess the amenability of the 48-well plates we compared the two methods but found that due to inadequate agitation of the 48-well plate the more time consuming Eppendorf preparation gave the most consistent and robust results. We did however manage to increase the efficiency of the assay and avoid the use of colorimetric techniques by incubating the preparation in an Eppendorf after which centrifugation steps gave the hemozoin pellet, which was dissolved in NaOH and transferred to a 48-well plate for analysis.

CHAPTER VII

Bibliography

7.0 Bibliography

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